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ESTUDIO DE LAS INTERACCIONES VIRUS-HOSPEDADOR: NUEVAS ALTERNATIVAS PROFILÁCTICAS FRENTE A FLAVIVIRUS

TESIS DOCTORAL

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"La vida es como un espejo: te sonríe si la miras sonriendo"

-Mahatma Gandhi-

RESUMEN

Las enfermedades infecciosas son causadas por microorganismos patógenos (bacterias, parásitos, hongos o virus) y pueden ser transmitidas directa o indirectamente de una persona a otra. Entre ellas destacan las enfermedades zoonóticas, que son aquellas que se transmiten entre animales vertebrados, incluidos los humanos. Éste es el caso del virus del Nilo Occidental (VNO), miembro del género *Flavivirus* (familia *Flaviviridae*), que es un patógeno neurotrópico que puede causar encefalitis y la denominada "fiebre del Nilo" en humanos. Como patógeno intracelular obligado, el VNO utiliza factores celulares para completar su ciclo de replicación, el cual está íntimamente asociado con el metabolismo lipídico celular. Así, se ha descrito que el VNO reorganiza las membranas intracelulares del retículo endoplasmático de las células infectadas para desarrollar plataformas adecuadas para una correcta replicación viral y su posterior expansión. Con estos antecedentes, el objetivo general de ésta tesis es profundizar en el conocimiento de los procesos celulares implicados en las interacciones patógeno-célula, y más concretamente en el papel de los lípidos celulares en la replicación vírica, con el fin de buscar dianas celulares para el desarrollo de estrategias antivirales innovadoras.

Los resultados obtenidos indican que la manipulación farmacológica del metabolismo lipídico celular es una potente estrategia alternativa para combatir el VNO; y que enzimas como la acetil-Coenzima A carboxilasa o la esfingomielinasa neutra, así como la vía metabólica de las proteínas de unión de elementos reguladores del esterol celular (*SREBPs*), son objetivos apropiados para el desarrollo de antivirales.

Es esperable que los conocimientos alcanzados contribuyan al estudio más detallado de las dinámicas de las interacciones lipídicas a lo largo del ciclo viral y, de este modo, al desarrollo de nuevas estrategias antivirales para combatir el VNO y otros flavivirus relacionados, tales como Usutu, dengue o Zika.

SUMMARY

Infectious diseases are caused by pathogenic microorganisms (bacteria, viruses, parasites or fungi) and can be spread, directly or indirectly, from one person to another. These include zoonotic diseases that are transmitted between vertebrates, including humans. This is the case of West Nile virus (WNV), a member of the genus *Flavivirus* (family *Flaviviridae*), which is a neurotropic pathogen and the causative agent of the so-called West Nile fever in humans and encephalitis. As an obligate intracellular pathogen, WNV uses cellular factors to complete its replication cycle, which is intimately associated to host cell lipid metabolism. Thus, it has been described that WNV rearranges intracellular membranes from the endoplasmic reticulum of infected cells to develop adequate platforms for an efficient viral replication and subsequent spread. Keeping this in mind, the general aim of this thesis was to achieve an accurate knowledge of the cellular processes involved in pathogen and host cell interactions, and more precisely in the role of the cellular lipids in viral replication, in order to search for cellular targets for the development of innovative antiviral strategies.

The obtained results indicate that the pharmacological manipulation of cellular lipid metabolism is an alternative potential strategy to combat WNV, and that enzymes, as the acetyl-CoA carboxylase (ACC) or the neutral sphingomyelinase (nSMase), as well as the cellular sterol regulatory element binding proteins (*SREBPs*) metabolic pathway, are suitable targets for antiviral development.

It is expected that the knowledge gained will contribute to a more detailed study of the dynamics of the lipids interactions throughout the viral cycle and, in this way, to the development of new antiviral strategies to fight WNV and other related flavivirus, such as Usutu, dengue, and Zika.

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ABBREVIATIONS

ACC: Acetyl-CoA Carboxylase
CDC: Centers for Disease Control and Prevention
CER: Ceramides
CERS: Ceramide Synthase
CM: Convoluted Membranes
DAAs: Direct Acting Antivirals
DENV: Dengue Virus
DHCR7: 7-dehydrocholesterol Reductase
dsRNA: doubled-strand RNA
E: Envelope
ECDC: European Centre for Disease Prevention and Control
EM: Electron Microscopy
ER: Endoplasmic Reticulum
FA: Fatty Acyls
FASN: Fatty Acid Synthase
GAGs: Glycosaminoglycans
GBS: Guillain-Barré syndrome
GGTase1: geranylgeranyl transferase type I
GLs: Glycerolipids
GPLs: Glycerophospholipids
HCV: Hepatitis C Virus
HMG-CoA reductase: hydroxyl methyl glutaryl-CoA reductase
ILCNC: International Lipid Classification and Nomenclature Committee
JEV: Japanese Encephalitis Virus
KUNV: Kunjin Virus
LDLR: low-density lipoprotein receptor
lyso-PC: lysophospholipids
M: Membrane
M4N: tetra-O-methyl nordihydroguaiaretic acid
MEDICA 16: 3,3,14,14-tetramethylhexadecanedioic acid
MVE: Murray Valley Encephalitis
NCR: Non-Coding Regions
NDGA: Nordihydroguaiaretic Acid
nm: nanometers
NS: Non-Structural
nSMase: neutral Sphingomyelinase
nt: nucleotide
NTPase: Nucleoside 5' Triphosphatase
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
pH: Potential of Hydrogen
PI: Phosphatidylinositol
PK: Polyketides
PRs: Prenol lipids
PS: Phosphatidylserine
RC: Replication Complex
RdRp: RNA-dependent RNA polymerase
RNA: Ribonucleic Acid
RSPs: Recombinant Subviral Particles (Partículas Subvirales Recombinantes)
RTPase: 5' RNA triphosphatase
RT-PCR: Real Time-PCR
SCLs: Saccharolipids
SL: Sphingolipids
SLEV: St. Louis Encephalitis Virus
SMase: Sphingomyelinase
SMS: Sphingomyelin Synthase
SPTLC: Palmitoyltransferase
SREBP: Sterol Regulatory Element Binding Proteins (Proteínas de unión de elementos reguladores del esterol celular)
STs: Sterol lipids
TOFA: 5-(tetradecyloxy)-2-furoic acid
USA: United States of America
USUV: Usutu Virus
UTR: Untranslated Regions
Vi: Virions
VNO: Virus del Nilo Occidental
Vp: Vesicle Packets
VZIKA: Virus de Zika
WNV: West Nile Virus
YFV: Yellow Fever Virus
ZIKV: Zika Virus

INTRODUCTION

1. West Nile Virus (WNV)

1.1 History and geographical distribution

The West Nile virus (WNV) belongs to the family *Flaviviridae* within the genus *Flavivirus*. This genus comprises over 70 viruses serologically classified into eight antigenic complexes, many of which, such as dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), or St. Louis encephalitis virus (SLEV) are important human and animal pathogens (Gould and Solomon 2008). Probably, the most well-known and representative virus belonging to this genus is the yellow fever virus, which gives name to the family ("flavi" comes from "flavus" that means yellow in Latin). Flaviviruses are **ar**thropod-**bo**rne **viruses** (arboviruses) that in many instances are zoonotic. These viruses are maintained in nature in complex infectious cycles that involve different vectors (mainly mosquitoes and ticks) and vertebrate hosts (Figure 1). Arthropod transmission of WNV includes uptake of virus by a vector during feeding on a viremic host, subsequent replication and dissemination of virus in the vector and, finally, transmission of the virus from the vector during feeding on an uninfected and susceptible host. WNV infections have been described in a wide variety of vertebrates, being the most important birds, especially wild birds, since they are the principal

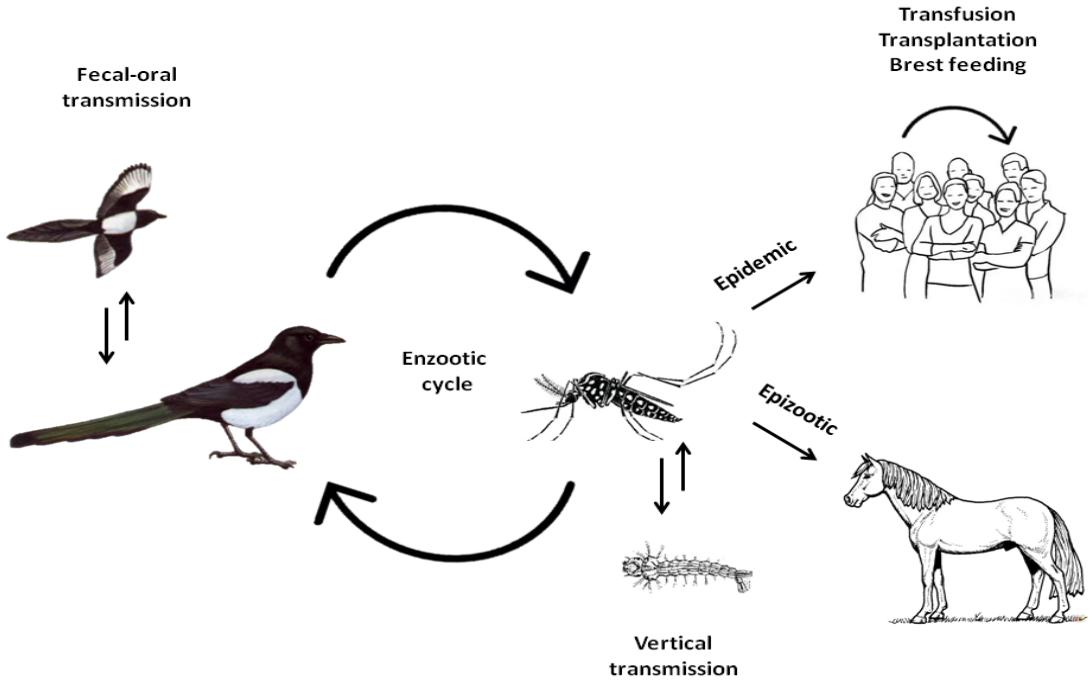


Figure 1: West Nile Virus transmission cycle. The WNV is transmitted to birds by infected mosquitoes and supplemented by bird-to-bird transmission. Vertical transmission among mosquitoes also occurs. Although the natural host of WNV are birds, it occasionally infects several other vertebrates including humans, in which non-vectorial transmission has also been documented.

hosts of the virus. The high and long-term viremia observed in wild birds allows transmission of WNV to its vectors, which are mainly ornitophilic mosquitoes of the *Culex* species. Migrations of birds have been shown to be instrumental in the introduction of WNV into previously unaffected areas. Beside birds, a broad range of other vertebrate species have been described as susceptible to WNV infection as well (rodents, bats, horses, or human and non-human primates, among others). In any case, the susceptibilities of birds to WNV infection differ between families, being the species belonging to the *Corvidae* family the more susceptible (Komar 2003). On the other hand, horses usually display an asymptomatic infection, with only 10%-12% of the infected animals showing signs of disease, being up to half of these cases fatal (van der Meulen, Pensaert et al. 2005). Likewise, in humans infections are usually asymptomatic, but occasionally WNV pathogenesis can range from mild illness such as fever, rash and joint pain, to more severe symptoms such as acute flaccid paralysis and fatal encephalitis (Martin-Acebes and Saiz 2012).

WNV was isolated for the first time in Uganda in 1937 from a febrile patient (Smithburn, Hughes et al. 1940) and, since then, it has caused infrequent outbreaks typically associated with mild febrile illnesses from the 1950s through the 1980s in Israel, Egypt, India, France and South Africa (Hurlbut, Rizk et al. 1956; George, Gourie-Devi et al. 1984; Jupp 2001; Balanca, Gaidet et al. 2009). Decrease in mosquito control efforts coupled with social factors, such as increased transportation and dense urbanization, have contributed to the emergence or reemergence of WNV. From the 1990s on, the frequency, severity, and geographic distribution of WNV outbreaks increased, and outbreaks of WNV encephalitis and meningitis affecting primarily adults hit Romania in 1996, Russia in 1999, and Israel in 2000 (Tsai, Popovici et al. 1998; Platonov, Shipulin et al. 2001; Weinberger, Pitlik et al. 2001). Later on, the number of affected European countries have increased, so that, during 2016 (Figure 2) 214 cases of West Nile fever in humans were reported in the EU, 93 in Romania, 68 in Italy, 43 in Hungary, 3 in Austria, 3 in Spain, 2 in Bulgaria, and one each in Cyprus and Croatia. In neighboring countries, 267 cases have been detected: 135 in Russia, 84 in Israel, 41 in Serbia, 2 in Syria, 2 in Turkey, and one each in Egypt, Tunisia and Ukraine (http://ecdc.europa.eu/en/healthtopics/west_nile_fever/pages/index.aspx). In Spain, the first human case was reported in Extremadura and more probably was an imported case from Portugal (Kaptoul et al., 2007). Later, the virus was isolated from a captive eagle (Jimenez-Clavero et al., 2008). In 2010, the first outbreaks were reported in horses in Andalusia, with 41 diagnosed cases and 10 deaths and a couple of non-fatal human cases (García-Bocanegra et al,

2010). Finally, along 2016 summer new outbreaks in horses were reported with three non-fatal human cases (http://ecdc.europa.eu/en/healthtopics/west_nile_fever/pages/index.aspx).

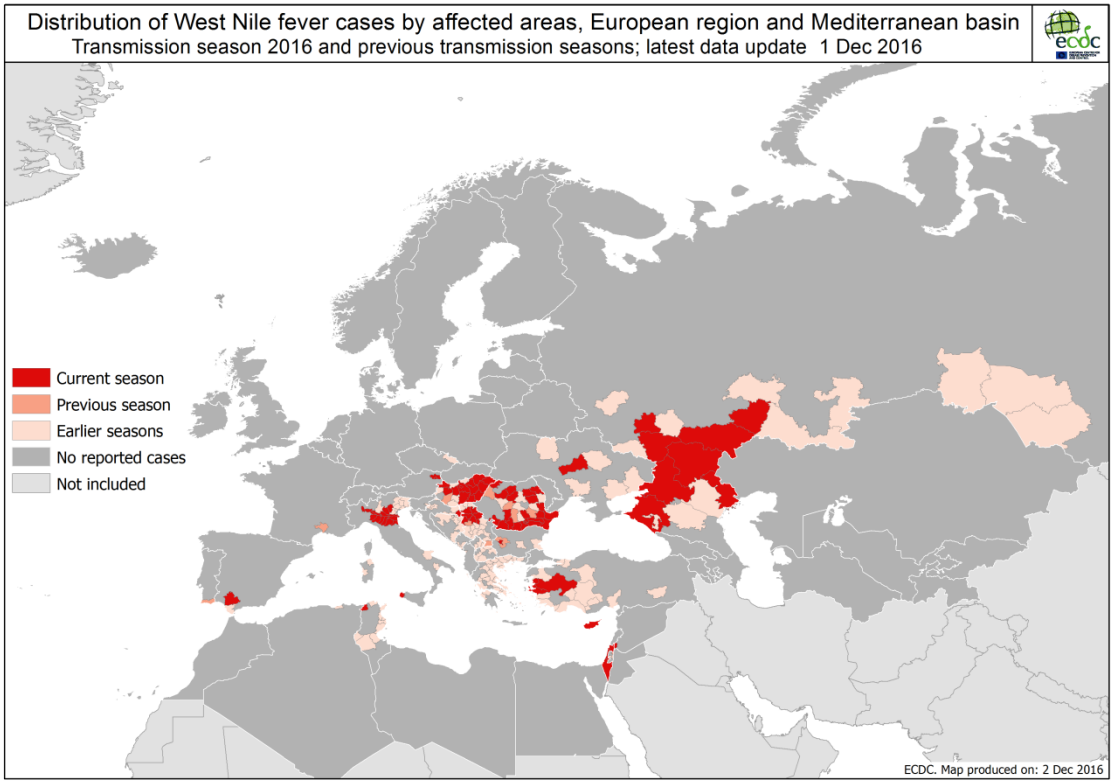


Figure 2: Human cases of West Nile fever Europe and the Mediterranean basin (transmission season 2016 and previous transmission seasons). Data collected from ECDC (http://ecdc.europa.eu/).

In the summer of 1999 WNV crossed the Atlantic and reached the Western Hemisphere, causing an outbreak in New York City (USA) that was responsible for 62 human cases of encephalitis, including seven deaths (Anderson, Andreadis et al. 1999; Lanciotti, Roehrig et al. 1999), 25 equine cases with nine deaths, and an enormous mortality of birds (Lanciotti, Roehrig et al. 1999). The nucleotide sequence of the invading strain was closely related to a 1998 isolate from Israel that have caused illness in geese (Lanciotti, Roehrig et al. 1999) and contained a mutation in the helicase gene that it has been proposed to cause high viremia and mortality in American crows (Brault, Huang et al. 2007). The current situation in the USA is that a total of 47 states and the District of Columbia have reported West Nile virus infections in people, birds, or mosquitoes in 2016 (Figure 3). Overall, 2.038 cases of West Nile virus disease in people have been reported to CDC during 2016. Of these, 1.114 (56%) were classified as neuroinvasive disease (such as meningitis or encephalitis) and 898 (44%) were classified as non-neuroinvasive disease (https://www.cdc.gov/westnile/index.html). Since its instruction in the USA, the virus rapidly spread to the neighbouring countries of Canada and Mexico. However, few human cases have been reported there, as well as in Central and South America.

In brief, in 80 years since its first isolation, the virus has propagated to a vast region of the globe and is now considered the most important causative agent of viral encephalitis worldwide.

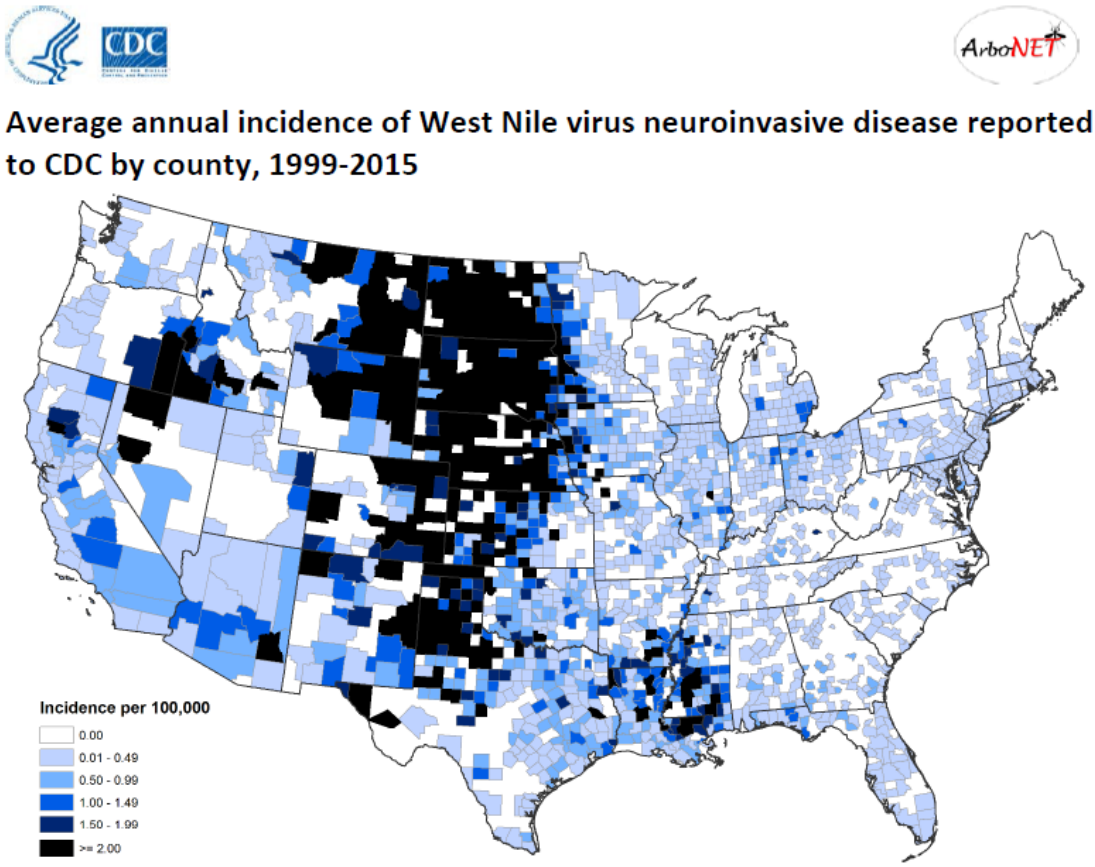


Figure 3: Human cases of West Nile fever in the United States of America (average annual incidence of WNV neuroinvasive disease report to CDC from 1999 to 2015. Data collected from CDC (https://www.cdc.gov/)).

1.2 Prevention and treatment of West Nile virus infection

Vaccines to protect humans against a number of flaviviruses such as tick-borne encephalitis virus, JEV or YFV, are available (Ulbert and Magnusson 2014; Hajj Hussein, Chams et al. 2015). Within the JEV serocomplex, JEV is the only flavivirus targeted by commercially available vaccines for humans, and immunization is performed using attenuated, inactivated or recombinant chimeric viruses (Halstead and Thomas 2010; Ishikawa, Yamanaka et al. 2014). Since 2001, several vaccines based on different approaches against WNV have been tested (Ishikawa, Yamanaka et al. 2014; Ulbert and Magnusson 2014), being some of them already approved for equine use. In any case, and although there is currently no licensed vaccine for humans, several clinical trials have been conducted exhibiting good immunogenic properties

(Ledgerwood, Pierson et al. 2011; Dayan, Bevilacqua et al. 2012; Durbin, Wright et al. 2013). One of the major drawbacks for vaccines implementation is their cost effectiveness. An evaluation of it conducted in 2006 concluded that introduction of WNV vaccine into the routine immunization program in the USA would not be worthy because of the relatively low incidence rates (Zohrabian, Hayes et al. 2006).

Regarding antiviral compounds, nowadays not a single one is available, which limits current treatments against WNV to only supportive care measures, such as intravenous antipyretics, fluids, respiratory support, and prevention of secondary infections. Despite this fact, intensive research during past decades has made significant progress in the design and development of several treatment methods for WNV infection. Current approaches and recent progress include therapeutic antibodies (Ben-Nathan, Lustig et al. 2003; Engle and Diamond 2003; Zhang, Vogt et al. 2009; Beigel, Nordstrom et al. 2010) and antiviral drugs targeting both viral replication and the host cell metabolism (Botting and Kuhn 2012; Lim and Shi 2013; Baharuddin, Hassan et al. 2014).

In this line, both viral (structural and non-structural) and host proteins constitute targets for developing antiviral therapeutics, as they are essential for WNV infection or replication. The search for antiviral compounds against WNV includes not only new drugs discovery, but also the repurposing of drugs approved for other purposes by testing inhibitory compounds that act on related flaviviruses, such as DENV or hepatitis C virus (HCV), as well as drugs used against non-related illness.

Regarding direct acting antivirals (DAAs) targeting viral replication, their viral target protein should be essential for the viral life cycle and should have a low rate of 'allowed' (non-lethal, but resistance-conferring) mutations. This second feature is especially important for flaviviruses because, as any other RNA viruses, their RNA polymerases do not have a proofreading activity (Boldescu, Behnam et al. 2017). Even though the mutation rate of these viruses leads to a relatively large proportion of non-functional progeny virions, it is an advantage for an efficient immune evasion and the development of drug resistances. The most promising viral targets are the NS3 protease and the NS5 polymerase, and, to a lesser degree, the E glycoprotein, the capsid protein, NS4B, NS3 helicase and NS5 methyltransferase. Other targets, such as NS5 guanylyltransferase and the NS3–NS5 interaction, currently seem to have limited potential (Boldescu, Behnam et al. 2017). It does exist a wide panel of options for DAAs development that are being evaluated, ranging from small molecules (natural and synthetic

compounds), antiviral peptides, and siRNAs to RNA-dependent RNA polymerase Inhibitors, among others (Botting and Kuhn 2012; Lim and Shi 2013; Baharuddin, Hassan et al. 2014).

Another antiviral approaches include targeting host cell metabolism and physiology and the modulation of the host immune system by using antiviral cytokines or cell enzymes inhibitors as potential therapeutics against WNV (Martin-Acebes, Merino-Ramos et al. 2014; Merino-Ramos, Vazquez-Calvo et al. 2015). These approaches have some advantages over DAAs: their effect is less prone to the development of resistance by mutations in the viral genome that frequently appear in RNA viruses, and they could confer broad-spectrum antiviral activity because certain host factors are usurped by a large number of different viruses. Nevertheless, broad-spectrum activity must be carefully confirmed on a case-by-case basis for each antiviral agent to avoid activation of other viral co-infections and, if possible, to cover co-circulating flaviviruses.

Considering the worldwide distribution of WNV and the evidence of its potential to change in pathogenicity and transmissibility, and being aware that prevention from insect bites remains the major defense against it, there is an urgent need to develop safe and effective novel vaccines and antiviral drugs against WNV infection. Therefore, a better understanding of the flavivirus life cycle and its pathogenesis is essential to develop effective strategies to combat these pathogens.

1.3 Molecular classification

First classifications of WNV were based on cross-neutralization reactions and revealed that WNV belongs to the Japanese encephalitis serocomplex. Recent advances on its molecular phylogeny disclosed the existence of up to five distinct genetic lineages of WNV (Figure 4), being lineage 1 and 2 the most clinically relevant. Lineage 1 is widely distributed and highly neurovirulent and is subdivided into three clades. Clade 1a includes African, European and American isolates (including NY99 isolate used during this doctoral thesis), clade 1b groups the Kunjin virus (KUNV) that is restricted to Australia, and clade 1c is found in Central Asia through the central highlands of India (Lanciotti, Ebel et al. 2002). Until recently, lineage 2 strains seemed to be confined enzootic in Africa and Madagascar and to be less neurovirulent than those included in lineage 1. However, lineage 2 strains were the cause of recent outbreaks in Europe in 2004 (Hungary) and 2008 (Austria) and, for the first time, caused a major epidemic in 2010 in Greece (Bakonyi, Ivanics et al. 2006; Chaskopoulou, Dovas et al. 2013). Since then, outbreaks involving WNV lineage 2 have been reported in several European countries including Italy, Serbia (including Serbia Novi Sad/12 isolate used along this doctoral thesis), and again in

Greece (Petric, Hrnjakovic Cvjetkovic et al. 2012; Chaskopoulou, L'Ambert et al. 2016). Some of these viral strains have appeared to become progressively more virulent for birds and humans (Malkinson, Banet et al. 2002). On the other hand, lineages 3 and 4 strains have been described from single isolates from Central Europe, but their taxonomic status and medical importance are unclear, whereas lineage 5 appears to be confined to India (Kramer, Styer et al. 2008).

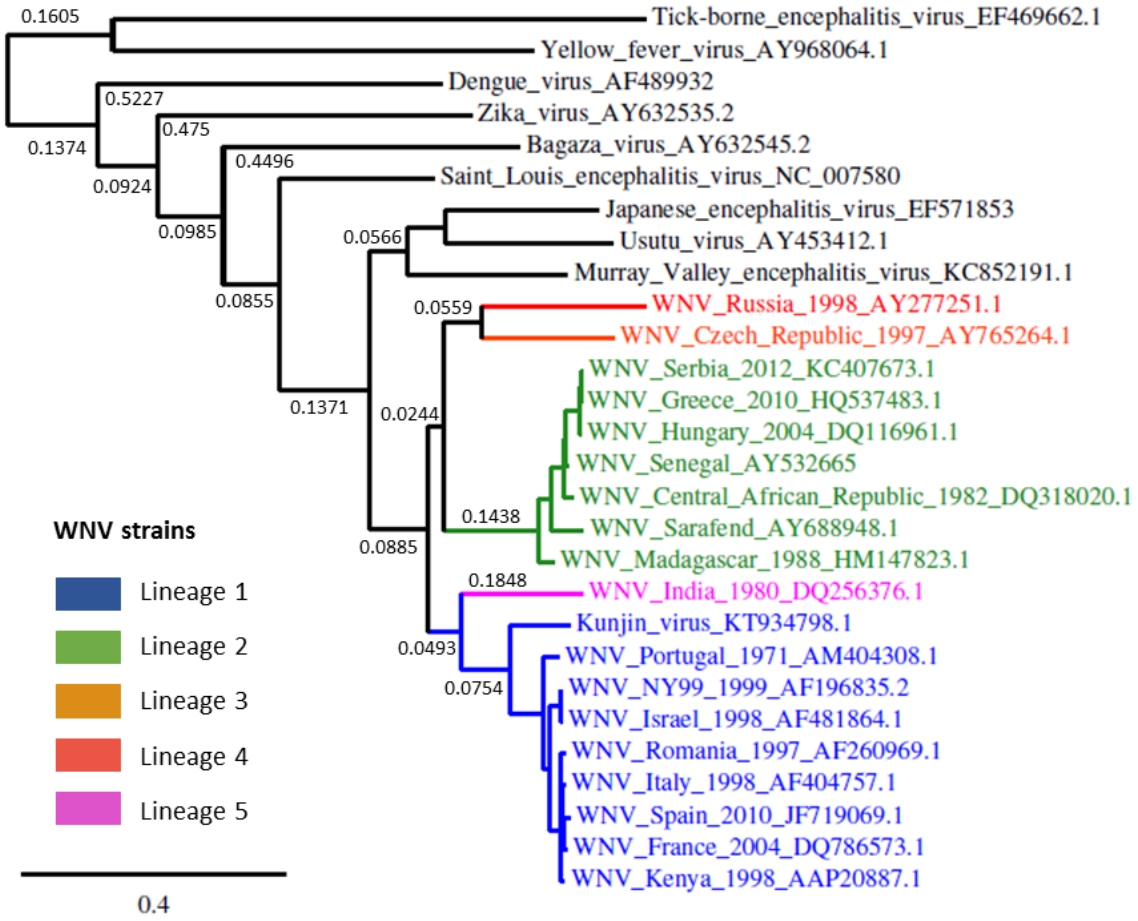


Figure 4: Representative phylogram showing the relationships between strains of the genus *Flavivirus*. Major WNV lineages are represented. Accession numbers are displayed in the tree as well as the year of isolation. The scale indicates 0.4 nt substitutions/site. The tree was based on complete NS5 nucleotide sequence, built from a multiple alignment using Clustal omega and Phylogeny.fr (Dereeper, Guignon et al. 2008).

1.4 Viral genome, structure, and physical properties of the virion

The WNV genome is constituted by a single stranded RNA molecule of positive polarity of about 11.000 nt in length (Lanciotti, Roehrig et al. 1999) that encodes a polyprotein in a single open reading frame flanked by two untranslated regions, UTR (Figure 5). This polyprotein is proteolitically processed by viral and cellular proteases, rendering 10 major viral proteins: 3

structural and 7 non-structural (Murray, Jones et al. 2008). This viral polyprotein contains multiple transmembrane domains that determine whether individual mature viral proteins are located on the cytoplasmic or luminal side of the endoplasmic reticulum (ER) membrane after cleavage from the polyprotein (Figure 5).

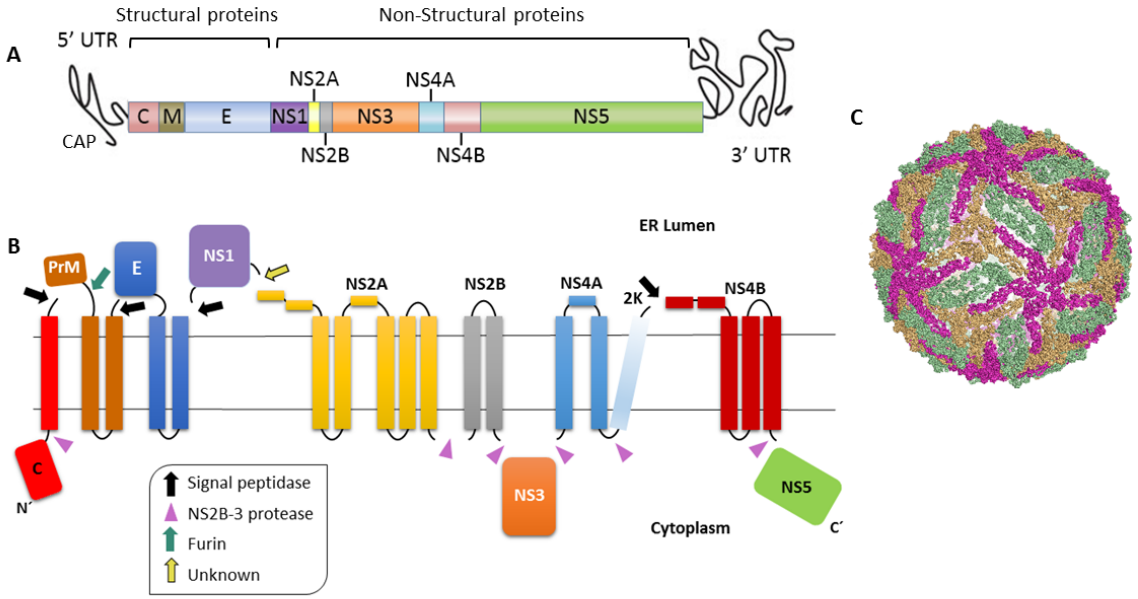


Figure 5: Genomic organization in WNV, polyprotein processing and virion structure. Schematic representation of WNV genome and particle. A) The viral genome is represented with its single ORF that encodes 3 structural (C, prM and E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) flanked by two untranslated regions at the 5' and 3' end of the molecule. It also has a cap at 5' end. B) Translation of the viral genome results in a polyprotein that is cleaved by cellular and viral proteases. The proposed topologies of the viral proteins with respect to the ER lumen and cytoplasm and the proteases responsible for their cleavages are indicated. Figure adapted from (Apte-Sengupta, Sirohi et al. 2014) C) WNV mature particle display a herringbone pattern of E glycoprotein dimers. The E glycoprotein monomers are colored in magenta, green, and orange to facilitate the interpretation of their distribution. Figure modified from (Martin-Acebes, Vazquez-Calvo et al. 2016).

Cryoelectron microscopy has unveiled that WNV particles are smooth and spherical (Figure 5), of about 50 nm in diameter, and contain an electron dense core, of about 30 nm diameter (Mukhopadhyay, Kim et al. 2003), composed by the viral genomic RNA coupled with the C (capsid) structural protein and surrounded by a lipid bilayer derived from the host cell. This lipid bilayer is flecked with the other two structural viral proteins, the E (envelope) and the M (membrane) proteins that form an outer shell of icosahedral symmetry (Mukhopadhyay, Kim et al. 2003). The E protein is the major surface protein of the viral particle (Figure 5), interacts with viral receptors, and mediates virus-cell membrane fusion. The M protein is a small proteolytic fragment of prM protein after being cleaved by a furin-like protease in the *trans*-Golgi network (Murray, Jones et al. 2008). The prM functions to protect the fusogenic E protein during transit through the acidic compartments of the secretory pathway, and its cleavage is an essential step for maturation of the virus into the infectious form (Brinton 2002).

The 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) function as coordinators of the intracellular aspects of virus replication, assembly, and modulation of the host defense mechanisms. NS1 is an essential cofactor for virus replication and inhibition of complement-mediated immune response (Avirutnan, Fuchs et al. 2010). NS3 contains serine protease, nucleoside 5' triphosphatase (NTPase), RNA helicase, and 5' RNA triphosphatase (RTPase) activities (Gorbalenya, Donchenko et al. 1989; Wengler 1991; Wengler 1993; Chappell, Stoermer et al. 2008), while NS2B serves as a cofactor for the protease activity of NS3 (Gayen, Chen et al. 2012). NS5 is necessary for genome replication and capping of emerging RNA, as it contains methyltransferase and RNA-dependent RNA polymerase (RdRp) domains (Brinton 2013; Lindenbach 2013). NS2A, NS2B, NS4A and NS4B are small, hydrophobic proteins, which structures and functions have not been yet fully characterized. Each of these proteins has several transmembrane domains and facilitates the assembly and/or anchoring of viral replication complexes on the ER membrane (Mackenzie, Khromykh et al. 1998; Westaway, Mackenzie et al. 2002). NS2A is involved in the production of intracellular virus-induced membranous structures, and in virion assembly (Kummerer and Rice 2002; Leung, Pijlman et al. 2008; Xie, Gayen et al. 2013). NS4A induces membrane rearrangement (Miller, Kastner et al. 2007; Carpp, Galler et al. 2011), whereas NS4B plays a major role on modulating the host immune response by suppressing the α/β interferon signaling (Munoz-Jordan, Laurent-Rolle et al. 2005).

The C, NS3, and NS5 proteins are located on the cytoplasmic side, while the prM, E, and NS1 proteins are in the lumen of the ER and, with the exception of short regions between transmembrane domains, the NS2A, NS2B, NS4A and NS4B proteins are located within the ER membrane bilayer (Figure 5) (Lindenbach 2013).

1.5 Replication cycle and virus cell host interactions

Viral penetration into the host cell is followed by genome uncoating, expression and replication, assembly of new virions and their egress (Figure 6). These steps occur in close association with cellular structures and membranes. In this line, several host lipid metabolic pathways have recently been identified and characterized as essential for viral replication. Figure 6 details the lipids involved at each step of the viral cycle (blue boxes).

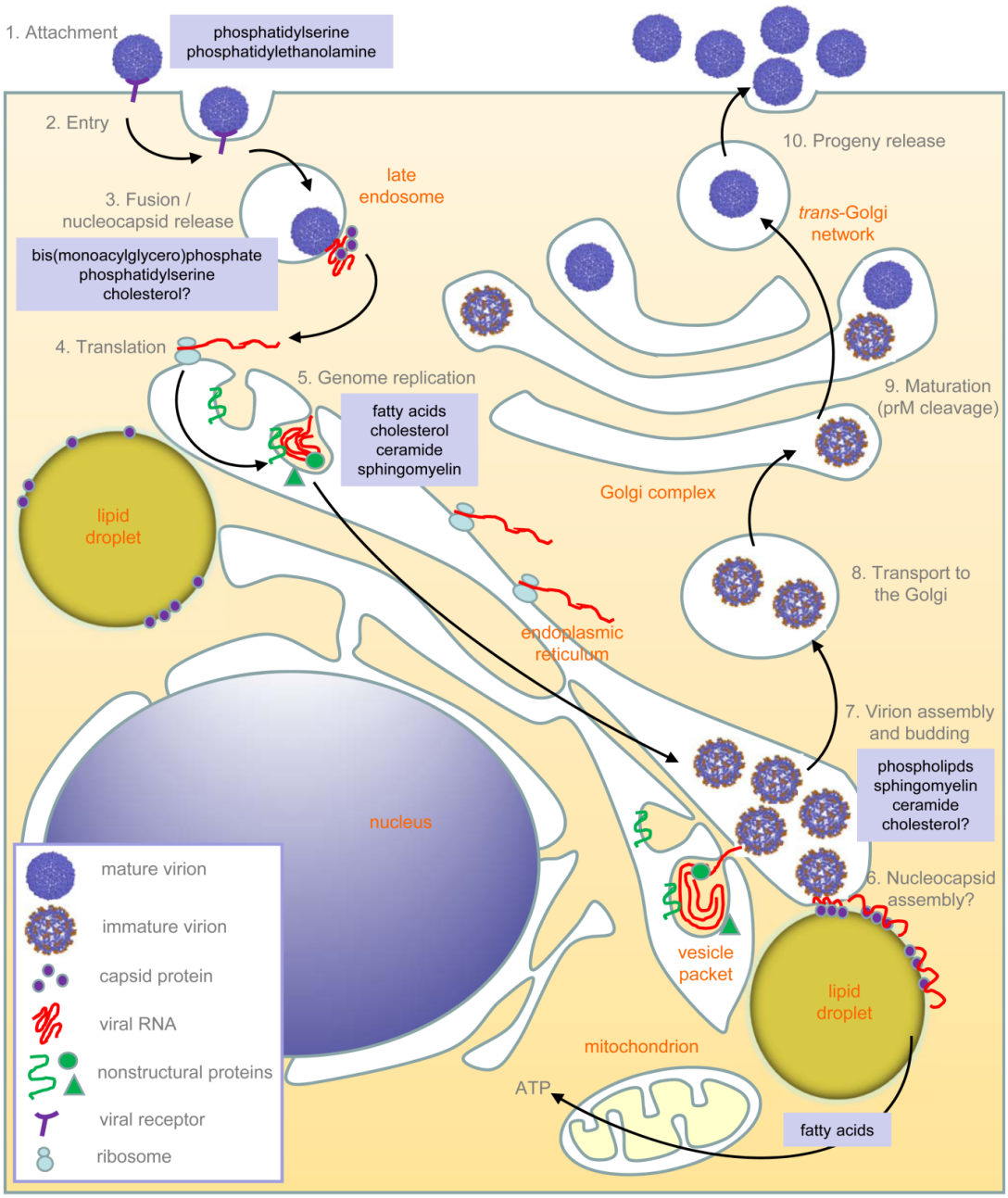


Figure 6: Flavivirus life cycle from the entry of the viral particle, through its genome replication and viral particle maturation, until their release. Note that the genome replication occurs on membranous structures formed by virus-induced perturbations of the ER membrane. Blue boxes highlight the lipids involved at each step. Image collected from (Martin-Acebes, Vazquez-Calvo et al. 2016).

The WNV infectious cycle starts when the envelope protein (E) engages unknown surface cellular receptors. Among the proposed receptors are glycosaminoglycans (GAGs), c-type lectins, and integrins (Chu and Ng 2004; Chu and Ng 2004). Then, the virus penetrates into the host cells by receptor mediated endocytosis. The low pH environment within the endosomal vesicles triggers viral fusion with the endosomal membrane leading to virion uncoating and release of the genomic RNA into the cytoplasm (Chu and Ng 2004; Pierson and Kielian 2013). The efficiency of the fusion process is likely affected by the specific lipid composition of both

the viral envelope and the target cellular membrane (Gollins and Porterfield 1986). Flavivirus replication begins when the viral (+) RNA genome is recognized as messenger RNA. Therefore, the genome can be translated by the host cell translation machinery to yield a single polyprotein.

The translation of this viral polyprotein occurs on the ER membranes, or on membranes derived from the ER (Figure 6), which is the site of glycerophospholipids, sterols, ceramides, and glycosphingolipids biosynthesis (Villareal, Rodgers et al. 2015). Flaviviruses, as any other plus-stranded RNA virus, remodel intracellular membranes developing organelle-like structures to create adequate platforms for viral replication (Miller and Krijnse-Locker 2008; Paul and Bartenschlager 2013). It is known that flavivirus replication and viral RNA synthesis occurs in close association with these virus-induced intracellular membranes structures that cover the genome amplification machinery, the so-called replication complex (RC), containing viral proteins, viral RNA, and probably host cell factors. RCs may promote efficient viral replication by anchoring the replication machinery in membrane compartments or by protecting it from host defense mechanisms (Mackenzie, Khromykh et al. 2007; Gillespie, Hoenen et al. 2010). Electron microscopy (EM) showed that flaviviral infection induces an extended network of modified ER membranes of various morphologies (Figure 6), including convoluted membranes, or vesicle packets (VP), that are invaginations of vesicle-like structures into the lumen of the ER (Welsch, Miller et al. 2009; Gillespie, Hoenen et al. 2010; Apte-Sengupta, Sirohi et al. 2014). In addition, these membranes increase the efficiency of viral processes by concentrating reactants and catalysts, and by scaffolding molecular interactions. The functions of these membranes in the biological processes are dictated by the physicochemical properties of their lipids component, including, but not limited to, membrane curvature and fluidity (Villareal, Rodgers et al. 2015). The localization of doubled-strand RNA intermediates (dsRNA), which are produced during replication of the viral RNA, as well as NS proteins, in these invaginations is consistent with the replication of the flavivirus genome in these structures (Westaway, Khromykh et al. 1999).

Once the genomic RNA has been replicated, it interacts with C proteins, and buds into the lumen as immature virus particles through the ER-derived membranes studded with prM and E proteins. During egress from infected cells, flaviviruses undergo dramatic structural changes through maturation steps in the Golgi complex, which are characterized by the formation of a herringbone arrangement of E proteins that lay flat against the surface of the virion, and cleavage of the prM protein by the cellular protease furin. The result is a relatively smooth, infectious mature virion (Mukhopadhyay, Kim et al. 2003).

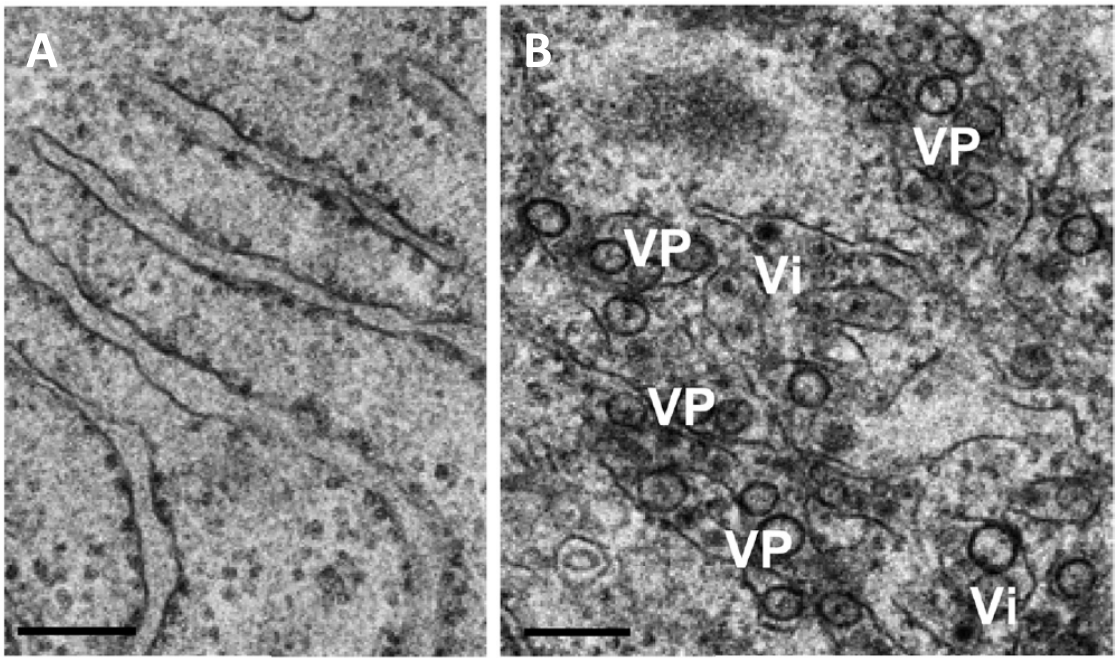


Figure 7: Intracellular membrane rearrangements in WNV infected cells compared to uninfected cells. Scale bars: 200 nm. Electron micrograph showing uninfected cells (A) and membrane alteration on WNV infected cells (B): vesicle packets (VP), and virions (Vi). Image modified from (Martin-Acebes, Merino-Ramos et al. 2014).

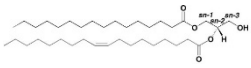
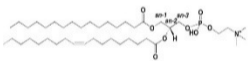
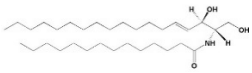
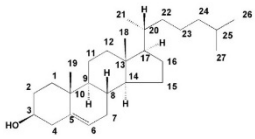
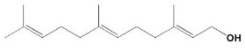
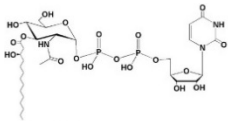
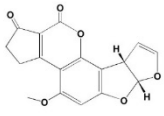
2. Lipids and flaviviruses

As commented above, host cell factors, noticeably lipids, play pivotal roles in viral infections. Although many host lipid metabolic pathways were characterized decades ago, it has been recently when they have been associated with viral infections.

Lipids display remarkable structural diversity, driven by factors such as variable chain length, a multitude of oxidative, reductive, substitutional and ring-forming biochemical transformations, as well as modifications with sugar residues and other functional groups of different biosynthetic origin. Within the cell, thousands of molecular lipid species exist that have been classified by the International Lipid Classification and Nomenclature Committee (ILCNC) into 8 categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Table) (Fahy, Cotter et al. 2011).

Lipids are the main components of cellular membranes where, as commented above, they play many roles due to their physicochemical properties (differences in the length of hydrocarbon chains, the degrees and locations of unsaturation, the sign, magnitude, location, and density of charges, the polarity, hydrogen-bonding capability, and size of the head-group) (Jarsch, Daste et al. 2016).

Table 1: Lipid categories and characteristics. The International Lipid Classification and Nomenclature Committee (ILCNC) divided lipids into 8 primary categories. Data collected from (Fahy, Subramaniam et al. 2005; Villareal, Rodgers et al. 2015).

Category	Basic Features
Fatty acyls (FA) 	Synthesized by chain elongation of an acetyl-CoA primer with malonyl-CoA (or methylmalonyl-CoA) groups that may contain a cyclic functionality and/or are substituted with heteroatoms. The FA structure represents the major lipid building block of complex lipids and therefore is one of the most fundamental categories of biological lipids. It is an energy source for the cell, important for signaling prostaglandin formation and membrane fluidity.
Glycerolipids (GLs) 	Enclose all glycerol-containing lipids. This category is dominated by the mono-, di- and tri-substituted glycerols, the most well-known being the fatty acid esters of glycerol (acylglycerols). Hydrolysis of these ester bonds, yields free FA that can undergo β-oxidation and produce ATP in the mitochondria. They are very abundant and important as membrane constituents, metabolic fuels, and signaling molecules.
Glycerophospholipids (GPLs) 	Shares with the GLs a common glycerol backbone. One alcohol of the core glycerol molecule forms a phosphate ester linkage. Phospholipids may be subdivided into distinct classes based on the nature of the polar "head group". The simplest GPL is phosphatidic acid. Additional substituents on the phosphate yield, respectively, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and, phosphatidylinositol (PI). They are ubiquitous in nature and are key components of the lipid bilayer of cells.
Sphingolipids (SLs) 	Complex family of compounds that share a common structural feature, a sphingoid base backbone that is synthesized *de novo* from serine and a long-chain fatty acyl-CoA, and then converted into ceramides (CER), phosphosphingolipids, glycosphingolipids, and other species, including protein adducts. CER are a major subclass of sphingoid base derivatives with an amide-linked fatty acid and are generally precursors of more complex SPs, such as sphingosines and sphingomyelin. Sphingosines are important for cell to cell signaling and cellular adherence to matrices, and are critical for protection of nerve cells via formation of the axon insulator. They interact with cholesterol to modulate lipid bilayer fluidity, especially at the plasma membrane.
Sterol lipids (STs) 	They have a fused four rings core structure and are subdivided on the basis of the number of carbons in the core skeleton. They are critical for membrane architecture and formation of lipid-ordered-domains ("lipid rafts"), which are important for signaling and dynamic movement of the cell. Additionally, the sterol lipid backbone is the precursor to steroid hormones and vitamin D. The archetype sterol found in most cellular membranes is cholesterol, which is critical for the entry and replication of many viruses.
Prenol lipids (PRs) 	Derived from condensation of isoprene subunits. Notably, prenol lipids are precursors to vitamins A, E, and K, and also function as antioxidants. Polyprenols are important for shuttling oligosaccharides across cell membranes.
Saccharolipids (SCLs) 	Similar to glycerolipids, but with a sugar backbone replacing the glycerol backbone.
Polyketides (PK) 	Derived from condensation of ketoacyl units, they are commonly found in bacteria and fungi. They are synthesized by multi-domain protein complexes composed of polyketide synthases, which bear similarities to fatty acid synthases (FASN). Many polyketides have anti-bacterial or anti-fungal properties, and some of them inhibit influenza virus.

The number of molecular species of lipids present in any cell membrane is much greater than would be required just to maintain a physical bilayer structure. This is because lipids, in addition to being a barrier to make compartments, perform a wide variety of other functions

including lipid dynamics, hydrophobic thickness, intrinsic bending tendency, surface electrostatics, phase behavior, and others (Epand 2007; Jarsch, Daste et al. 2016). Membranes are involved in fusion and fission, as well as protrusion and invagination, exocytosis and endocytosis, and cell division, in which the planar bilayer membrane transforms into other shapes, at least transiently and locally (Kimura, Jennings et al. 2016).

Formation of these curved membrane structures can be facilitated by certain lipid molecular species as well as by proteins (Epand 2007); (Villareal, Rodgers et al. 2015). Head-group size and tail saturation influence the surface area that a lipid occupies in a membrane. A lipid with a small head-group (PE) and unsaturated tails forms a conical shape, whilst large head-groups (PI) with saturated tails form an inverted cone, and large head-groups with saturated or singly unsaturated side chains have a cylindrical shape (PC). Lipids with small head-groups will tend to localize in regions of negative curvature, whereas species with large head-groups localize in regions of positive curvature (Figure 8).

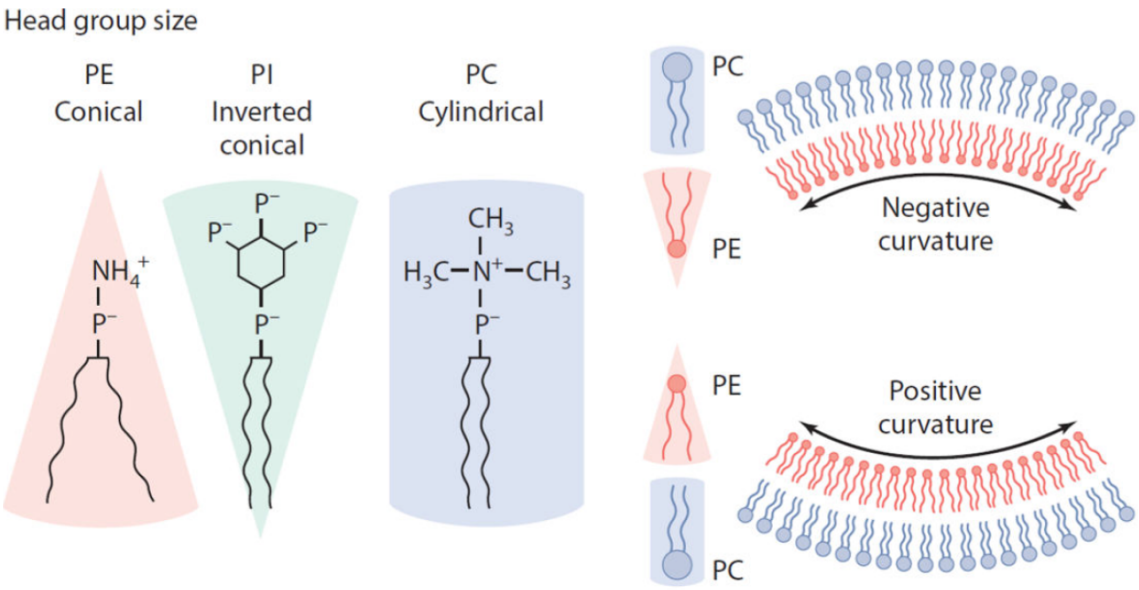


Figure 8: Formation of curve membranes. The accumulation of certain lipids can induce membrane bilayer bending. Figure from (Villareal, Rodgers et al. 2015).

What is more, lipids play physical, chemical, and biological regulatory roles in living systems. Studies of signaling lipids are revealing that many biological processes are dependent rather on specific lipid species than on the entire group of lipids of a particular class (Kimura, Jennings et al. 2016). Even so, most of the studies made on this subject are still crude (Lee, Lin et al. 2008; Munger, Bennett et al. 2008). In fact, even small changes in the structure of the molecules can result in significant changes in biological function. The influence of the alteration of the

content of a broad range of lipid metabolites in cells infected with a variety of human viral pathogens has been proved (Mackenzie, Khromykh et al. 2007; Soto-Acosta, Mosso et al. 2013). Besides, it is known that lipid membranes are structural components of all enveloped virions and that they serve as replication platforms (Heaton and Randall 2011). Thus, since viruses lack the appropriate machinery to perform their own lipid synthesis, they are forced to co-opt for enzymes involved in this process to complete their replication cycles. Among these enzymes, the most relevant are those related to FA, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) (Martin-Acebes, Blazquez et al. 2011); those related to SL, such as serine palmitoyltransferase (SPTLC), ceramide synthase (CERS) (Aktepe, Pham et al. 2015), sphingomyelin synthase (SMS) (Martin-Acebes, Gabande-Rodriguez et al. 2016) and sphingomyelinase (SMase); and those to ST, such as 7-dehydrocholesterol reductase (DHCR7), hydroxyl methyl glutaryl-CoA reductase (HMG-CoA reductase) and geranylgeranyl transferase type I (GGTase1) (Mackenzie, Khromykh et al. 2007) (Figure 9).

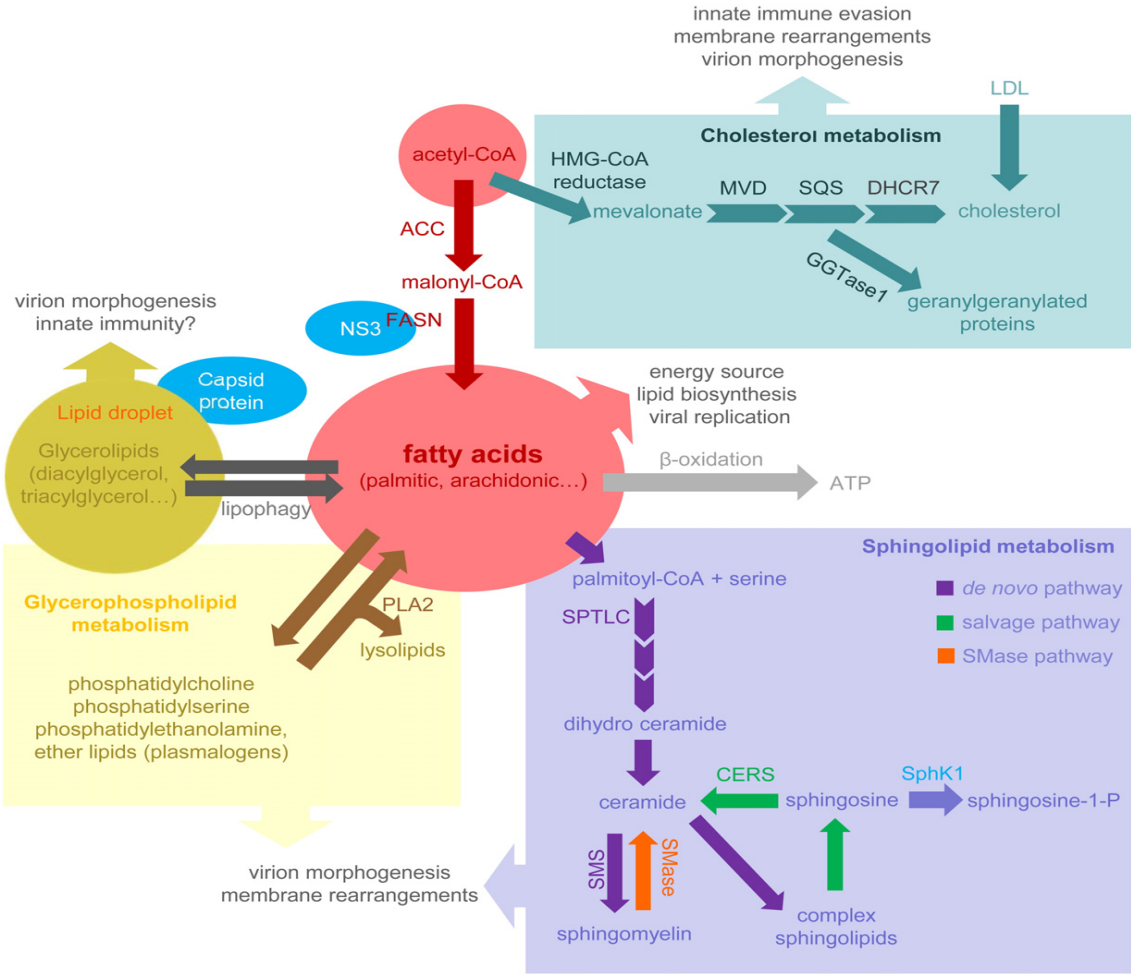


Figure 9: Flavivirus interaction with cell lipid metabolism. Flaviviruses exert an influence on fatty acids, cholesterol, sphingolipids, glycerophospholipids and lipid droplets (Martin-Acebes, Vazquez-Calvo et al. 2016).

It has been proposed that the lipid rearrangements that viruses exert over cell lipid metabolism are aimed to create an adequate environment for proper viral replication and particle biogenesis. In addition, viruses can dramatically orchestrate a profound reorganization of the host cell lipid metabolism to adequate cellular function to their own requirements (Figure 9). Furthermore, the localization of viral gene expression, replication of the viral genome, and viral assembly to membranes compartmentalizes them, thus protecting them from host defense systems and gaining competence of viral processes by concentrating reactants. In any case, plenty of questions are still unanswered to unveil the tight relationship between lipids and viral infections and, because of that, this was the focus of our research on this topic.

3. Usutu Virus (USUV)

As shown in Figure 4, Usutu virus (USUV) is also a *Flavivirus* closely related to WNV (Bakonyi, Gould et al. 2004). Both viruses share common genetics and antigenic features, both have an African origin, and both have lately emerged in Europe (Ashraf, Ye et al. 2015).

Figure 10: Map showing the geographic locations of USUV-related epidemiological studies on birds and mosquitoes in Europe. Data from (Ashraf, Ye et al. 2015; Santini, Vilibic-Cavlek et al. 2015; Cadar, Luhken et al. 2017).

USUV was first described in 1959 as a part of a study of viral prevalence in arthropods in South Africa, near the Usutu river, from where it took its name (Woodall 1964). Since then, it presence has been described in several African countries, including Senegal, Central African Republic, Nigeria, Uganda, Burkina Faso, Cote d' Ivore, and Morocco (Nikolay, Diallo et al. 2011). USUV was reported to be circulating only in Africa until 2001, when it emerged in Central Europe (Weissenbock, Kolodziejek et al. 2002). Although retrospective studies have demonstrated that USUV was circulating in Italy, at least, since 1996 (Weissenbock, Bakonyi et al. 2013). From that moment on, USUV has been detected in several European countries (Figure 10) generally associated with episodes of avian mortality (Ashraf, Ye et al. 2015; Santini, Vilibic-Cavlek et al. 2015; Cadar, Luhken et al. 2017; Cadar, Maier et al. 2017).

USUV vectors are ornithophilic mosquitoes, mainly of the *Culex* species, and its natural hosts are birds, mostly blackbirds (*Turdas merula*). Nevertheless, from time to time, it can infects other vertebrates as humans, where infections are mainly asymptomatic, although patients may sporadically present symptoms ranging from fever, rash, or jaundice to meningoencephalitis (Pecorari, Longo et al. 2009; Santini, Vilibic-Cavlek et al. 2015). Currently no vaccine or therapy exists against this pathogen.

4. Zika virus (ZIKV)

Another medically relevant *Flavivirus* (Figure 4) that recently has been a worldwide headline new is the Zika virus (ZIKV). As the above described WNV and USUV, ZIKV is a small enveloped single stranded positive RNA virus (Wang, Thurmond et al. 2017). The virus was isolated for the first time in April 1947 from the serum of a febrile sentinel rhesus monkey that was caged in the canopy of the Zika forest, near Lake Victoria (Dick, Kitchen et al. 1952), hence its name.

Mosquitoes, mainly of the *Aedes* species, are the arthropod vectors of the ZIKV natural transmission cycle (Saiz, Vazquez-Calvo et al. 2016). Early studies indicated that non-human primates were the primary vertebrate hosts, with occasional participation of humans in the transmission cycle, even in highly enzootic areas (Haddow and Dick 1948; Haddow, Williams et al. 1964); however, nowadays humans are their main hosts. ZIKV can also infect other vertebrates like rodents, birds, reptiles, goats, sheep, and cattle, although their role on viral spread seems to be negligible (Saiz, Vazquez-Calvo et al. 2016).

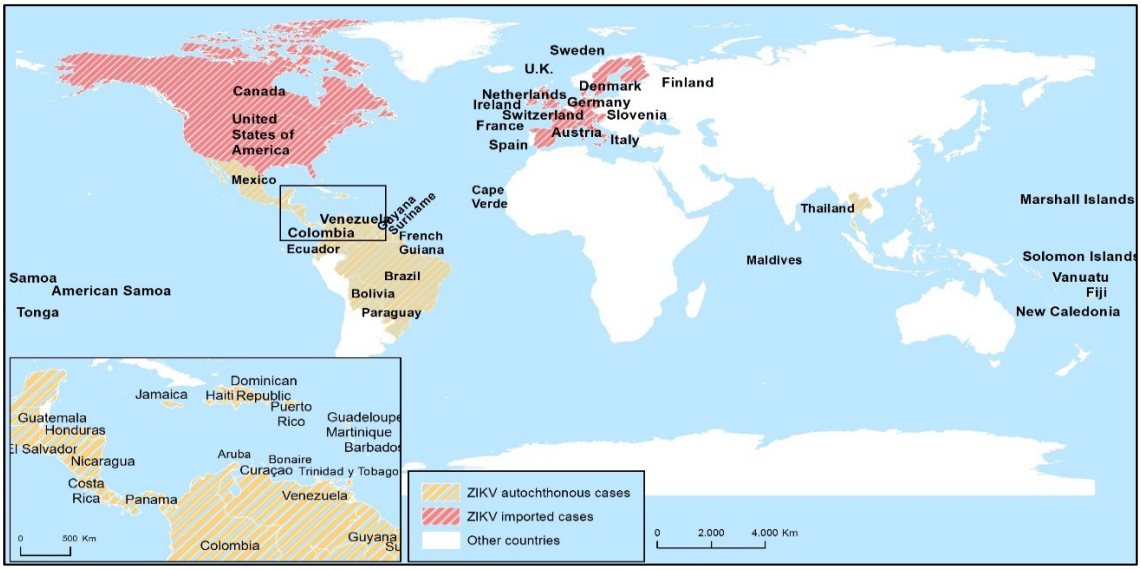


Figure 11: Map showing worldwide autochthonous and imported ZIKV human cases during the 2015-2016 global outbreak (Saiz, Vazquez-Calvo et al. 2016).

The virus was mainly confined to the African continent until it was detected in south-east Asia the 1980's, then in the Micronesia in 2007 and, more recently in the Americas in 2015 (Figure 11) where it has caused a global outbreak infecting millions of people (Saiz, Vazquez-Calvo et al. 2016). ZIKV infection was characterized by causing a mild disease presented with fever, headache, rash, arthralgia, and conjunctivitis, with exceptional reports of an association with Guillain-Barré syndrome, GBS (Ansar and Valadi 2015; Blazquez and Saiz 2016). However, during the recent outbreaks in America, a twenty times increase in the number of cases of microcephaly in fetus and newborns, and an astonishing number of GBS associated cases have been linked to ZIKV infection (Blazquez and Saiz 2016). As for any flavivirus, and beside the great effort made by the scientific community during recent months (Saiz and Martin-Acebes 2017), there are neither specific drugs licensed nor vaccines against ZIKV.

OBJECTIVES

El principal objetivo de esta tesis doctoral era el obtener un conocimiento más detallado del proceso celular implicado en las interacciones de los flavivirus con la célula hospedadora y, más concretamente, en el papel que juegan los lípidos celulares en la replicación viral, para la búsqueda de dianas celulares antivirales

Los objetivos específicos fueron:

1 La evaluación del impacto de la infección por el VNO en el contenido lipídico de la célula hospedadora.
2 La caracterización de la composición lipídica de la envuelta del VNO.
3 La determinación del papel de ciertos lípidos específicos en la biogénesis del VNO.
4 El análisis del efecto de inhibidores de la vía de la biosíntesis de ácidos grasos en la infección con el VNO y con el VUSU.
5 El análisis del efecto de inhibidores de la vía de las proteínas de unión a elementos reguladores del esterol (SREBP) en la infección con el VNO y con el VZIKA.

The main goal of this thesis was to gain a detailed knowledge of the cellular processes involved in flavivirus and host cell interactions, and more precisely in the role of the cellular lipids in viral replication, to search for cellular antiviral targets.

The specifics goals of this thesis were:

1 To evaluate the impact of WNV infection on the lipid content of the host cell.

2 To characterize the lipid composition of the WNV envelope.

3 To determine the role of specific lipids in WNV biogenesis.

4 To analyze the effect of inhibitors of the cellular fatty acid biosynthetic pathway on the infection by WNV and USUV.

5 To analyze the effect of inhibitors of the cellular sterol regulatory element binding proteins (SREBP) pathway on infection by WNV and ZIKV.

ARTICLES

INTRODUCTION TO THE ARTICLES PROVIDED FOR THE DOCTORAL THESIS AND PhD FELLOW CONTRIBUTION.

Articles provided for the doctoral thesis

Article I: Martín-Acebes MA*, **Merino-Ramos T***, Blázquez AB, Casas J, Escribano-Romero E, Sobrino F, Saiz JC. "The composition of West Nile virus lipid envelope unveils a role of sphingolipid metabolism in flavivirus biogenesis". J Virol. 2014 Oct;88(20):12041-54. doi: 10.1128/JVI.02061-14. PMID: 25122799 (***Martín-Acebes MA and Merino-Ramos T equally contributed to this article**.)

Article II: Merino-Ramos T, Vázquez-Calvo Á, Casas J, Sobrino F, Saiz JC, Martín-Acebes MA. **"**Modification of the host cell lipid metabolism induced by hypolipidemic drugs targeting the acetyl coenzyme A carboxylase impairs West Nile virus replication". Antimicrob Agents Chemother. 2015 Oct 26;60(1):307-15. doi: 10.1128/AAC.01578-15. PMID: 26503654.

Article III: Merino-Ramos T, Jiménez de Oya A, Saiz JC, Martín-Acebes MA. "Antiviral activity of Nordihydroguaiaretic acid and its derivative tetra-O-methyl nordihydroguaiaretic acid against West Nile virus and Zika virus"**.** Antimicrob Agents Chemother. 2017 May 15 pii: AAC.00376-17. doi: 10.1128/AAC.00376-17.

Breve introducción a los artículos y su contribución al campo/Brief introduction to the articles included and its contribution to the field.

Artículo I

Martín-Acebes MA*, **Merino-Ramos T***, Blázquez AB, Casas J, Escribano-Romero E, Sobrino F, Saiz JC. "The composition of West Nile virus lipid envelope unveils a role of sphingolipid metabolism in flavivirus biogenesis". J Virol. 2014 Oct;88(20):12041-54. doi: 10.1128/JVI.02061-14. PMID: 25122799 (***Martín-Acebes MA and Merino-Ramos T equally contributed to this article**.)

Como se describía en la introducción, distintos pasos del ciclo de vida viral dependen de su asociación con las membranas plasmáticas, las cuales a menudo se encuentran modificadas durante la infección. De hecho, varios estudios han demostrado que la biología celular de los flavivirus está íntimamente relacionada con las membranas intracelulares, y el requerimiento de lípidos específicos, como el colesterol y los ácidos grasos se ha documentado como esencial para la replicación viral y la biogénesis de partículas infecciosas (Martin-Acebes, Vazquez-Calvo et al. 2016). Una estrategia atractiva e innovadora para inhibir patógenos virales es manipular dianas del hospedador de las cuales dependen. Para desarrollar estrategias antivirales que inhiban esos procesos de infección por flavivirus a través de efectos estrechamente dirigidos a la síntesis lipídica del hospedador, primero se deben definir los lípidos específicos implicados en la infección por flavivirus y por tanto se necesita un mayor entendimiento elemental de las funciones de los lípidos en la replicación y biogénesis. El conocimiento detallado de cómo moléculas específicas están implicadas en la replicación viral, permitiría un diseño racional de antivirales dirigidos a dianas selectivas del hospedador. Esto puede ser llevado a cabo reduciendo la toxicidad para el hospedador a través de la identificación de lípidos que puedan ser modulados sin interferir con las funciones del hospedador, y/o actuando sobre pasos intermedios de la síntesis lipídica, metabolismo o tráfico de sustancias, para los cuales los virus tienen más sensibilidad que la célula hospedadora. Aunque la composición de las envueltas lipídicas de unos pocos virus ha sido caracterizada, en el **Artículo I** se caracteriza por primera vez la composición de la envuelta del VNO. El artículo describe que las envueltas están enriquecidas en esfingolípidos y que muestran niveles reducidos de fosfatidilcolina (un glicerofosfolípido). La consiguiente inhibición de la nSMasa (la cual cataliza la hidrólisis de esfingomielina a ceramida) reduce la liberación de viriones de flavivirus y de partículas subvirales recombinantes, confirmando la relevancia de los esfingolípidos en la biogénesis del

VNO. El impacto en la infección por VNO sobre dos componentes de las membranas celulares (glicerofosfolípidos y esfingolípidos), así como la comparación de la envuelta lipídica de los viriones del VNO y de las partículas subvirales recombinantes, revelaron que ambos tienen una composición lipídica única. El **Artículo I** también describe cómo la composición lipídica celular se ve alterada por la infección viral.

Durante el desarrollo de esta investigación realicé, junto al Dr. Martín-Acebes, la mayoría del trabajo experimental desarrollado, por lo que ambos somos **co-primeros autores**, tal y como se reseña en el artículo, y contribuí al diseño de una parte significativa de los experimentos. Llevé a cabo las infecciones de células (Vero, HeLa y C6/36) con virus (VNO y USUTU) y su tratamiento con concentraciones crecientes de distintos fármacos (GW4869, Spiroepoxido y glutatión) determinando la toxicidad de los mismos en las distintas células utilizadas y titulando las progenies víricas obtenidas. Purifiqué las partículas subvirales recombinantes (RSPs) y los viriones. Obtuve y cuantifiqué el ARN de doble cadena mediante inmunofluorescencia en células infectadas, tratadas o no con fármaco. Así mismo, obtuve el ARN viral y lo analicé por RT-PCR cuantitativa. Finalmente llevé a cabo parte del análisis de los datos y contribuí a la escritura del artículo detallando los resultados obtenidos.

Article I

Martín-Acebes MA*, **Merino-Ramos T***, Blázquez AB, Casas J, Escribano-Romero E, Sobrino F, Saiz JC. "The composition of West Nile virus lipid envelope unveils a role of sphingolipid metabolism in flavivirus biogenesis". J Virol. 2014 Oct;88(20):12041-54. doi: 10.1128/JVI.02061-14. PMID: 25122799 (***Martín-Acebes MA and Merino-Ramos T equally contributed to this article**.)

As described in the introduction, different steps of the virus life cycle depend on its association with the cytoplasmic membranes, which are often modified during infection. In fact, several studies have shown that the cellular biology of flaviviruses is intimately linked to modifications of the intracellular membranes, and the requirement of specific lipids, such as cholesterol and fatty acids, has been documented as essential for virus replication and infectious particle biogenesis (Martin-Acebes, Vazquez-Calvo et al. 2016). An attractive and innovative strategy to inhibit viral pathogens is to manipulate the host targets on which they depend. To develop antiviral strategies to inhibit these flaviviral processes through tighter focused effects on specific host lipid synthesis, the specific lipids that are implicated in flavivirus infection must first been defined, and, thus, a better elemental understanding of the lipid roles in viral replication and biogenesis is needed. Knowing in detail how specific lipid molecules are involved in viral replication, a rational design of selective host-targeted antivirals could be performed. This can be achieved by reducing host toxicity through the identification of lipids that can be modulated without interfering with host cell functions, and/or by targeting steps in lipid synthesis, metabolism, or trafficking for which viruses have a greater sensitivity than the host cell. Although the lipid composition of the viral envelopes of a few distinct viruses had been characterized, in **Article I** the WNV envelope composition was characterized for the first time. The article describes that envelopes were enriched in sphingolipids and showed reduced levels of phosphatidylcholine (a glycerophospholipid). Subsequent inhibition of nSMase (which catalyzed the hydrolysis of sphingomyelin into ceramide) reduced the release of flavivirus virions and virus-like particles, confirming the relevance of sphingolipids in the biogenesis of WNV. The impact of WNV infection on two important components of the cellular membranes (glycerophospholipids and sphingolipids), as well as the comparison of the lipid envelope of WNV virions and recombinant-subviral particles, revealed that they have a unique composition. **Article I** also described how the cellular lipid composition is altered by the viral infection.

Along the development of this research, together with Dr. Martín-Acebes, we developed most of the experimental work detailed. For this reason, we have both the co-authorship of the publication. As it is described in the article, I contributed to the experimental design. I made the cells infections (Vero, HeLa and C6/36) with viruses (WNV and USUV) and its treatment with increasing concentrations of different drugs (GW4869, Spiroepoxide and Glutathione). Previously, I determined drug toxicity in the same cell lines and afterwards, I made the viral titration. I purified recombinant subviral particles (RSPs) and virions, and obtained and quantified dsRNA intermediates by immunofluorescence in infected cells treated or not with drugs. Likewise, I made viral RNA extractions and analyzed them by quantitative RT-PCR. Finally, I did a part of the data analysis and contributed to the article writing, detailing the obtained results.

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The Composition of West Nile Virus Lipid Envelope Unveils a Role of Sphingolipid Metabolism in Flavivirus Biogenesis

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ABSTRACT

West Nile virus (WNV) is an emerging zoonotic mosquito-borne flavivirus responsible for outbreaks of febrile illness and meningoencephalitis. The replication of WNV takes place on virus-modified membranes from the endoplasmic reticulum of the host cell, and virions acquire their envelope by budding into this organelle. Consistent with this view, the cellular biology of this pathogen is intimately linked to modifications of the intracellular membranes, and the requirement for specific lipids, such as cholesterol and fatty acids, has been documented. In this study, we evaluated the impact of WNV infection on two important components of cellular membranes, glycerophospholipids and sphingolipids, by mass spectrometry of infected cells. A significant increase in the content of several glycerophospholipids (phosphatidylcholine, plasmalogens, and lysophospholipids) and sphingolipids (ceramide, dihydroceramide, and sphingomyelin) was noticed in WNV-infected cells, suggesting that these lipids have functional roles during WNV infection. Furthermore, the analysis of the lipid envelope of WNV virions and recombinant virus-like particles revealed that their envelopes had a unique composition. The envelopes were enriched in sphingolipids (sphingomyelin) and showed reduced levels of phosphatidylcholine, similar to sphingolipid-enriched lipid microdomains. Inhibition of neutral sphingomyelinase (which catalyzes the hydrolysis of sphingomyelin into ceramide) by either pharmacological approaches or small interfering RNA-mediated silencing reduced the release of flavivirus virions as well as virus-like particles, suggesting a role of sphingomyelin-to-ceramide conversion in flavivirus budding and confirming the importance of sphingolipids in the biogenesis of WNV.

IMPORTANCE

West Nile virus (WNV) is a neurotropic flavivirus spread by mosquitoes that can infect multiple vertebrate hosts, including humans. There is no specific vaccine or therapy against this pathogen licensed for human use. Since the multiplication of this virus is associated with rearrangements of host cell membranes, we analyzed the effect of WNV infection on different cellular lipids that constitute important membrane components. The levels of multiple lipid species were increased in infected cells, pointing to the induction of major alterations of cellular lipid metabolism by WNV infection. Interestingly, certain sphingolipids, which were increased in infected cells, were also enriched in the lipid envelope of the virus, thus suggesting a potential role during virus assembly. We further verified the role of sphingolipids in the production of WNV by means of functional analyses. This study provides new insight into the formation of flavivirus infectious particles and the involvement of sphingolipids in the WNV life cycle.

West Nile virus (WNV) is a mosquito-borne flavivirus that is distributed worldwide and that is responsible for recurrent outbreaks of febrile illness and encephalitis. The virus is maintained in nature in an enzootic infectious cycle between birds and mosquitoes, which act as its vectors, although it can also infect multiple vertebrate hosts, including horses and humans (1, 2). The continuing spread of WNV due to a variety of ecological factors, combined with the lack of specific therapeutics or vaccines for human use, makes the identification of the viral and host processes that control the biology of this pathogen important to improve the design of specific antiviral strategies (3).

As a flavivirus, WNV is an enveloped plus-strand RNA virus (1, 2). A feature that it shares with other plus-strand RNA viruses is replication in the cytoplasm of infected cells in tight association with intracellular membrane rearrangements (4). In the case of flaviviruses and, hence, WNV, the membranes associated with virus replication are from the endoplasmic reticulum (ER) (5). Even more, flavivirus particles are assembled by invagination and budding of the ER membrane into the lumen of this organelle, so the ER also provides the membrane source for WNV envelopment (6). Although the lipid composition of the viral envelopes of a few distinct viruses has been characterized (7–10), to our knowledge,

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the molecular composition of the flavivirus envelope has not been analyzed. Only certain lipids in the flavivirus envelope (cholesterol and phosphatidylserine) have been identified, and these have been identified by functional studies rather than by analytical approaches (11, 12).

Lipids are the main components of cellular membranes, playing key roles in viral infections by acting as signaling molecules, as well as by determining the physical properties of the membranes, such as fluidity, thickness, or shape (13, 14). In fact, the strong manipulation of cellular lipid metabolism by different viruses has recently been documented (15–17). Accordingly, enrichment in specific lipids contributes to the generation of a membrane curvature adequate for the correct assembly of the replication complex or virus budding, and the cone-shaped or inverted cone-shaped lipids, which can govern membrane bending, budding processes, or fusion events, are of particular interest (18–20). Although differences in the lipid requirements between viruses of the same family have been noted (21), the dependence on certain lipids, such as fatty acids (15, 22, 23) or phosphatidylinositol 4-phosphate (PI4P) (21, 24), can be shared by unrelated enveloped and nonenveloped viruses. These observations suggest that each virus creates its own characteristic cellular microenvironment for replication by developing specialized virus-induced organelle-like structures within infected cells (4, 24, 25). Regarding flaviviruses, major rearrangements of cellular lipid metabolism have been observed in dengue virus (DENV)-infected cells (26, 27), and several studies have highlighted the importance of both fatty acids and cholesterol for WNV and DENV infection (22, 28–31), as well as a lack of a requirement for PI4P (21, 29).

In this study, we have analyzed the effect of WNV infection on the cellular content of 11 lipid classes. Our results revealed an increase in the cellular content of multiple lipid species upon WNV infection, which is consistent with major lipid metabolic changes in WNV-infected cells. The proportions of these metabolites in the envelope of WNV virions and virus-like particles were also determined, unveiling a unique composition of the viral envelope and pointing to specific roles of some of these lipids for virus assembly. Supporting this view, a direct link between specific lipids (sphingolipids) and WNV biogenesis was found by means of functional analyses.

MATERIALS AND METHODS

Cells, viruses, infections, and virus titrations. All infectious virus manipulations were performed in our biosafety level 3 (BSL-3) facilities. The origins of WNV strain NY99, Usutu virus (USUV) strain SAAR-1776, and Sindbis virus (SINV) have been previously described (32). Vero and HeLa cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified minimum essential medium (DMEM) supplemented with 2 mM glutamine, penicillin-streptomycin, and 5 or 10% fetal bovine serum, respectively. Cells of a HeLa cell line stably transfected with plasmid pcDNA 3.1 (+) (Invitrogen, Carlsbad, CA) encoding the 25 last amino acids of the WNV NY99 C protein followed by the sequence of premembrane/membrane (prM) and envelope (E) proteins (HeLa3-WNV cells) were obtained by limiting dilution and grown in complete culture medium supplemented with 500 μg/ml G-418 (T. Merino-Ramos, A. B. Blázquez, E. Escribano-Romero, R. Cañas-Arranz, F. Sobrino, J. C. Saiz, and M. A. Martín-Acebes, submitted for publication). This line constitutively secreted WNV recombinant subviral particles (RSPs). C6/36 mosquito cells were cultured in M3 medium supplemented with 4 mM glutamine, gentamicin, penicillin-streptomycin, nonessential amino acids, amphotericin B, and 10% fetal bovine serum at 28°C without CO_2. For infections in liquid medium, the viral inoculum was incubated with cell monolayers for 1 h at the temperature appropriate for cell growth, and then the inoculum was removed and fresh medium containing or not containing 1% fetal bovine serum was added. The viral titer was determined at 24 h postinfection (p.i.) for WNV and USUV and 8 h p.i. for SINV by plaque assay on Vero cells (29, 32, 33). Cell-associated virus was extracted from infected cells by three cycles of freeze and thaw and titrated as described above. The multiplicity of infection (MOI) used in each experiment was expressed as the number of PFU/cell and is indicated in the corresponding figure legend.

Antibodies. Mouse monoclonal antibodies against the WNV envelope (E) protein (Millipore, Temecula, CA), β-actin (Sigma, St. Louis, MO), and GM130 (ECM Biosciences, Versailles, KY) and rabbit sera against WNV M protein (Imgenex, San Diego, CA), calnexin (StressMarq Biosciences Inc., Victoria, Canada), mannosidase II (Millipore), and sphingomyelin phosphodiesterase 3 (SMPD3), also named neutral sphingomyelinase 2 (nSMase2; ECM Biosciences), were used as primary antibodies. Anti-mouse or anti-rabbit IgG coupled to Alexa Fluor 594 or 488 (Invitrogen, Carlsbad, CA) and goat anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase (Pierce Biotechnology, Rockford, IL) were used as secondary antibodies.

Drug treatments. Brefeldin A (BFA), golgicide A (GCA), GW4869, and glutathione were from Sigma. Spiroepoxide was from Santa Cruz Biotechnology (Santa Cruz, CA). BFA, GCA, GW4869, and spiroepoxide stock solutions were prepared in dimethyl sulfoxide, while glutathione was directly dissolved in culture medium. Working concentrations of GW4869 were prepared as described previously (34). Control cells were treated in a parallel manner with the same amount of drug vehicle. For estimation of RSP release, the medium from HeLa3-WNV cells was replaced by serum-free medium containing the drugs. Cells were incubated for 4 h, and the amount of RSPs released to the culture medium was determined by an enzyme-linked immunodot assay using a monoclonal antibody directed against WNV E glycoprotein, as described below. In the case of virus infections, drugs were added after the first hour of infection, when the viral inoculum was replaced by medium containing 1% fetal bovine serum. The lack of toxicity of drug concentrations during the assays was evaluated by determination of the cellular ATP content with a CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI).

siRNA experiments. HeLa or HeLa3-WNV cells were transfected in serum-free culture medium with 100 nM endoribonuclease-prepared small interfering RNAs (esiRNAs) against human *SMPD3* (MISSION; Sigma) or an equivalent amount of MISSION small interfering RNA (siRNA) universal negative control 1 (MISSION; Sigma) using an siRNA transfection reagent (MISSION; Sigma), as indicated by the manufacturer. To estimate the release of WNV RSPs, the culture medium of HeLa3-WNV cells was replaced by fresh serum-free medium, and the amount of RSPs in the culture medium was determined after 4 h of incubation by an enzyme-linked immunodot assay. For viral infections, transfected cells were infected (MOI, 1 PFU/cell) as described above for infections in liquid medium, and subsequent experiments were performed at 48 h posttransfection. The extent of siRNA silencing was analyzed by Western blotting (29, 35) using specific antibodies against nSMase2.

Purification of RSPs and virions. HeLa3-WNV cells were incubated for 48 h in serum-free medium (to avoid the possible interference of serum with subsequent lipid determinations), and RSPs were purified by sucrose gradient centrifugation essentially as described previously (36). Briefly, culture medium from HeLa3-WNV cells was clarified by centrifugation at 15,000 × *g* for 30 min at 4°C and then centrifuged through a 20% sucrose cushion for 3.5 h at 112,000 × *g* at 4°C. The pellet containing RSPs was resuspended in phosphate-buffered saline (PBS), loaded onto a 12-ml 20 to 60% linear sucrose gradient, and centrifuged at 256,000 × *g* for 18 h at 4°C. For purification of WNV particles, HeLa cells were infected with WNV (MOI, 5 PFU/cell) in serum-free medium and incubated for 48 h. The culture medium was cleared of cell debris by centrifugation at 850 × *g* for 15 min at 4°C and centrifuged through a 20% sucrose cushion at 112,000 × *g* for 3.5 h at 4°C. The pellet containing the virions was

loaded onto a six-step discontinuous 20 to 60% sucrose gradient and centrifuged at 55,500 × *g* for 18 h at 4°C. Gradients were fractioned from the top, and the E-protein content of each fraction was determined by enzyme-linked immunodot assay as described below. Only the fraction with the highest E-protein content was selected for lipid analyses.

Immunofluorescence and confocal microscopy. Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min and processed for immunofluorescence as described previously (29, 35). Cells were observed using a Leica TCS SPE confocal laser scanning microscope and an HCX PL Apo ×63/1.4 oil immersion objective. Images were acquired using Leica advanced fluorescence software and processed with Adobe Photoshop CS2 software (Adobe Inc., San Jose, CA). The optical slice thickness for all confocal images displayed was 1 airy unit.

Transmission electron microscopy. Negative staining of RSPs was performed in dialyzed samples containing the peak amount of E protein within the gradient, as described previously (36). For immunolabeling and negative staining, samples were adsorbed to ionized collodion-carbon-coated grids, washed with PBS, and blocked with 10% fetal bovine serum in PBS for 5 min. The grids were incubated with primary antibodies diluted in 5% fetal bovine serum in PBS for 30 min, washed five times with PBS, and incubated with protein A coupled to 5-nm colloidal gold diluted in 5% fetal bovine serum in PBS for 30 min. Samples were then fixed with 1% glutaraldehyde in PBS for 5 min, washed three times with bidistilled water, and negatively stained with 1% uranyl acetate for 2 min. HeLa or Vero cells infected with WNV were fixed in 4% paraformaldehyde–2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 24 h p.i. and processed for electron microscopy as described previously (29, 35). Samples were examined using a JEOL JEM-1010 electron microscope (JEOL, Japan) operated at 80 kV, and images were acquired using a digital camera (4K64K TemCam-F416; Tietz Video and Image Processing Systems GmbH, Gauting, Germany).

Enzyme-linked immunodot assay. Cell culture medium (usually 5 to 10 μl) was adsorbed to a nitrocellulose membrane by vacuum using a Bio-Dot apparatus (Bio-Rad, Hercules, CA). For quantification of the amount of RSPs in the culture medium and in order to verify that samples were within the linear range, a standard curve was prepared using different dilutions. The membrane was blocked with 3% skimmed milk in PBS and incubated with monoclonal anti-E antibody diluted in 1% skimmed milk in PBS. After three washes with PBS, the membranes were incubated with secondary antibodies coupled to horseradish peroxidase and washed, and proteins were detected by chemiluminescence using ImageQuant LAS 4000 mini equipment (GE Healthcare, Buckinghamshire, United Kingdom).

Quantitative RT-PCR. Viral RNA was extracted with a NucleoSpin viral RNA isolation kit (Macherey-Nagel, Düren, Germany). The number of viral RNA copies was determined by quantitative reverse transcription-PCR (RT-PCR) (37) and is given as the number of genomic equivalents corresponding to the number of PFU/ml by comparison with the amount of RNA extracted from previously titrated samples (38).

Lipid analysis. HeLa cells were infected or not infected (mock infected) with WNV (MOI, 50 PFU/cell) in serum-free medium as described above for infections in liquid medium, detached from the flasks, and resuspended in PBS at 24 h p.i. The number of cells in each sample was determined, and aliquots containing 2.7×10^6 cells were subjected to lipid extractions. The amount of protein in each sample was also determined by the Bradford assay. Lipids were extracted using a modified Bligh and Dyer protocol. Cell pellets were resuspended in 100 μl of PBS and 750 μl of a methanol-chloroform (1:2, vol/vol) solution containing 0.01% butylated hydroxytoluene (BHT) and internal standards (1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoserine, 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphoethanoamine, and 1-heptade canoyl-2-hydroxy-*sn*-glycero-3-phosphoserine, 200 pmol each, from Avanti Polar Lipids). Samples were extracted at 48°C overnight and centrifuged at 13,000 × *g* for 5 min. Then, the supernatant was transferred to a new vial, evaporated to dryness, and stored at −20°C in an argon atmosphere until the analysis of glycerophospholipids (39). On the other hand, 100 μl of PBS and 750 μl of a methanol-chloroform (2:1, vol/vol) solution containing 0.01% BHT and internal standards (*N*-lauroyl-D-*erythro*-sphingosine, *N*-lauroyl-D-*erythro*-sphingosylphosphorylcholine, and D-glucosyl-β-1,1′-*N*-lauroyl-D-*erythro*-sphingosine, 200 pmol each, from Avanti Polar Lipids) were added to the cell pellets. Samples were extracted at 48°C overnight and cooled, 75 μl of 1 M KOH in methanol was added, and the mixture was incubated for 2 h at 37°C. Following addition of 75 μl of 1 M acetic acid, samples were centrifuged at 13,000 × *g* for 5 min and the supernatant was transferred to a new vial, evaporated to dryness, and stored at −20°C in an argon atmosphere until the analysis of sphingolipids (40).

Lipids were measured with an Acquity ultraperformance liquid chromatography (UPLC) system (Waters, USA) connected to a time-of-flight (TOF; LCT Premier XE) detector controlled with Waters/Micromass MassLynx (v.4.1) software. An Acquity UPLC BEH C_8 column (particle size, 1.7 μm; 100 mm by 2.1 mm; Waters, Ireland), a flow rate of 0.3 ml/min, and a column temperature of 30°C were used. The mobile phase was methanol with 1 mM ammonium formate and 0.2% formic acid (solution A)–water with 2 mM ammonium formate and 0.2% formic acid (solution B). Gradient elution started at 80% solution A, was increased to 90% solution A over 3 min, was held for 3 min, was increased to 99% solution A over 9 min, and was held for 3 min. Initial conditions were attained in 2 min, and the system was stabilized for 3 min. The acquisition range of the TOF detector was *m/z* 50 to 1,500, the capillary voltage was set to 3.0 kV, the desolvation temperature was 350°C, and the desolvation gas flow rate was 600 liters/h (41).

Lipid identification was confirmed by the analysis of one sample from each group with an ultra-high-performance liquid chromatography system (Accela) coupled to a Thermo Fisher Scientific LTQ Orbitrap Velos mass spectrometer (MS) controlled with Thermo Fisher Scientific/Xcalibur software using the same column and eluted with the following conditions: gradient elution started at 85% solution A, was increased to 90% solution A over 9 min, was held for 2 min, was increased to 99% solution A over 6 min, and was held for 2 min. Initial conditions were attained in 2 min, and the system was stabilized for 3 min. The acquisition range of the Orbitrap detector was *m/z* 200 to 100, the source voltage was set to 3.5 kV, the capillary temperature was 350°C, the sheath gas flow rate was 50 liters/h, the auxiliary gas flow rate of 20 liters/h, and the sweep gas flow rate was 2 liters/h (42).

Individual chromatographic peaks of distinct lipid species were isolated from full-scan MS spectra when the theoretical exact masses of the lipid species were selected. The spectra were extracted from a database that was previously generated using the spectrum simulation tool of Micromass MassLynx software on the basis of our previous results (39). Then, a list of possible candidates fitting the specific exact mass was generated using formula determination tools (elemental composition search) of Micromass MassLynx software. The elemental number was restricted to include C, H, O, N, and P. The formula constraints were as follows: C, H, and O were ≥1, P was ≥0, and N was ≥1, following the nitrogen rule. The number of double-bond equivalents (DBEs) was set to be between −0.5 and 15.0. The search was based on single mass analysis and considered only the *m/z* value of the monoisotopic peak. Positive identification of the lipids was based on accurate mass measurement with an error <5 ppm, a low i-Fit parameter in the spectrum window, and its relative retention time by liquid chromatography compared to that of the standard (±2%) (39). The following lipids from Avanti Polar Lipids were used: 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine, 1-hexadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphocholine, 1-hexadecanoyl-2-(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoyl)-*sn*-glycero-3-phosphocholine, 1-(9*Z*-octadecenoyl)-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-(1*Z*-octadecenyl)-2-(9*Z*-octadecenoyl9-*sn*-glycero-3-phosphocholine, 1-octadecanoyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine, 1-hexadecanoyl-2-(9*Z*-octadecenoyl)-

sn-glycero-3-phospho-L-serine, *N*-hexadecanoyl-D-*erythro*-sphingosine, *N*-hexadecanoyl-D-*erythro*-sphingosylphosphorylcholine, and D-glucosyl-β-1,1′-*N*-hexadecanoyl-D-*erythro*-sphingosine In addition, identifications of lipids were made by searching their molecular weights against entries in the Lipid MAPS database. As mentioned above, this identification procedure was confirmed by the analysis of selected samples using Thermo Fisher Scientific Orbitrap analyzers (resolution, 30,000 full width at half maximum) (42).

The data presented are the products of 4 to 5 independent lipid extractions and determinations.

Annotation of lipid species. Glycerophospholipids were annotated as lipid subclass and total fatty acyl chain length:total number of unsaturated bonds. Plasmalogens (P) were annotated as described above, except that P was added. Sphingolipids were annotated as lipid subclass and total fatty acyl chain length:total number of unsaturated bonds. If the sphingoid base residue was dihydrosphingosine (dihydro sphingomyelin or dihydro ceramide), the lipid class contains the prefix dh (dihydro).

Data analysis. Analysis of variance (ANOVA) was performed with the SPSS (v.15) statistical package (SPSS Inc., Chicago IL), with Bonferroni's correction applied for multiple comparisons. Nonparametric comparisons were performed using the statistical package GraphPad Prism (v.2.01; GraphPad Software Inc., La Jolla, CA). Data are presented as the mean ± standard deviation (SD). Differences with P values of <0.05 were considered statistically significant.

RESULTS

Alteration of lipid content in WNV-infected cells. To analyze the effect of WNV infection on the lipid content of the host cell, HeLa cells were infected or not infected at a high multiplicity of infection (MOI) and processed for lipid analysis at 24 h p.i. At this time point, infectious virus release into the culture medium was already detected (Fig. 1A). When the cells were observed by transmission electron microscopy, they exhibited intracellular membrane rearrangements associated with flavivirus replication and assembly, such as membrane sacs containing small virus-induced vesicles, referred to as vesicle packets (VPs) (5, 29, 43), as well as electron-dense virions (Fig. 1B). As expected, these rearrangements were not observed in control uninfected cells. Eleven lipid classes (seven glycerophospholipids [GPLs] and four sphingolipids [SLs]) were included in the analysis. The GPLs analyzed were phosphatidylcholine (PC), 1-alkenyl,2-acylglycerolphosphocholine (referred to as plasmalogen-PC [p-PC; plasmenylcholine]), 2-acylglycero-3-phosphocholine (lyso-PC), phosphatidylethanolamine (PE), plasmalogen-PE (p-PE), 2-acylglycero-3-phosphoethanolamine (lyso-PE), and phosphatidylserine (PS). The four SLs analyzed were ceramide (CER), dihydro-CER (dhCER), sphingomyelin (SM), and dihydro-SM (dhSM). A tendency for an increase in the content of most of the lipid classes analyzed per cell (except for lyso-PE, a minor lipid class present in the extracts) was noticed in WNV-infected cells (Fig. 1C). In fact, statistically significant increases in the amounts of PC (1.7-fold), p-PC (1.9-fold), lyso-PC (1.9-fold), p-PE (1.6-fold), CER (1.8-fold), dhCER (1.3-fold), and SM (1.4-fold) were noted when infected cells were compared to uninfected cells. Among the 11 lipid classes analyzed, a total of 162 different molecular species were identified in HeLa cells (the complete list of molecular species identified in this and subsequent analyses is included in Data Set S1 in the supplemental material), with 54 (33.3%) molecular species being significantly increased upon WNV infection (Fig. 1D). Interestingly, although a tendency for a reduction of some lipid species was found, at the level of resolution of this analysis, no statistically significant reduction in the content of individual lipid species could be determined, so we assumed that no major modifications in the level of the rest of the lipid species occurred in infected cells. The molecular species significantly increased in infected cells included 44 GPLs (13 PCs, 9 p-PCs, 3 lyso-PCs, 5 PEs, and 14 p-PEs) and 10 SLs (5 CERs, 1 dhCERs, and 4 SMs). Therefore, WNV infection markedly alters the lipid metabolism of host cells.

GPL and SL content of the envelope of flavivirus virions and RSPs. The lipid composition of viral envelopes can provide novel clues to understand the biology of these pathogens (7, 9, 10). Therefore, we studied the GPL and SL content of the viral envelope of WNV. Since the coexpression of flavivirus structural glycoproteins (premembrane/membrane [prM/M] and envelope [E]) results in the assembly and secretion of a sort of virus-like particle termed a recombinant subviral particle (RSP) that provides a noninfectious model system to study the assembly and secretion of flavivirus particles (44, 45), the analysis of the lipid composition of RSPs was included in this study. A stable HeLa clone (HeLa3-WNV cells) expressing WNV structural glycoproteins (Fig. 2A) that constitutively secrete WNV RSPs of about 30 nm in diameter into the culture medium (Fig. 2B) was used as a source of RSPs. The identity of these structures was confirmed by immunogold electron microscopy using an antibody against the E glycoprotein (Fig. 2B inset). In this cell line, immunofluorescence and confocal analysis revealed that the WNV envelope glycoprotein colocalized with markers of the ER (calnexin) and the Golgi complex (mannosidase II) (Fig. 2C and D). The molecular weights of the proteins were analyzed by Western blotting using anti-E and anti-M antibodies (Fig. 2E). As expected, using anti-E antibody, a single band (about 53 kDa [46]) was observed in samples containing purified RSPs. In the case of anti-M antibody, two bands, one band compatible with prM protein and the other band compatible with mature M protein (about 20 and 6 kDa, respectively [46]), were observed. These results confirmed the correct expression of WNV glycoproteins by cells of the HeLa3-WNV cell line.

Enzyme-linked immunodot assay is faster than Western blotting and allows the analysis of up to 96 samples at once. This kind of assay has successfully been applied to quantify the amount of RSPs released into the culture medium by other flaviviruses (47). Thus, a dot blot assay was used to detect the WNV RSPs released into the culture medium from HeLa3-WNV cells (Fig. 2F). To further validate this methodology, the amount of RSPs released into the culture medium upon treatment with the Golgi complex-disturbing agents brefeldin A (BFA) or golgicide A (GCA) was studied. Treatment of HeLa3-WNV cells with BFA or GCA impaired the release of RSPs into the culture medium (Fig. 3A). The quantification of the blots revealed that BFA or GCA at concentrations of ≥5 μM significantly inhibited the release of RSPs into the culture medium without exerting noticeable toxic effects on the cells (Fig. 3B). Immunofluorescence analysis of BFA- or GCA-treated cells confirmed the disruption of the Golgi complex architecture that these drugs induced (Fig. 3C). Golgi complex disassembly was concomitant with the accumulation of E glycoprotein inside the cells (Fig. 3C), an observation consistent with a reduction in the release of RSPs into the culture medium observed in Fig. 3A and B. Overall, these observations are consistent with previous data showing that flavivirus RSPs, as well as infectious virions, are assembled by budding into the ER and then traffic through the secretory pathway (44, 45). Therefore, in the HeLa3-WNV cell model, the lipid envelope of RSPs is acquired

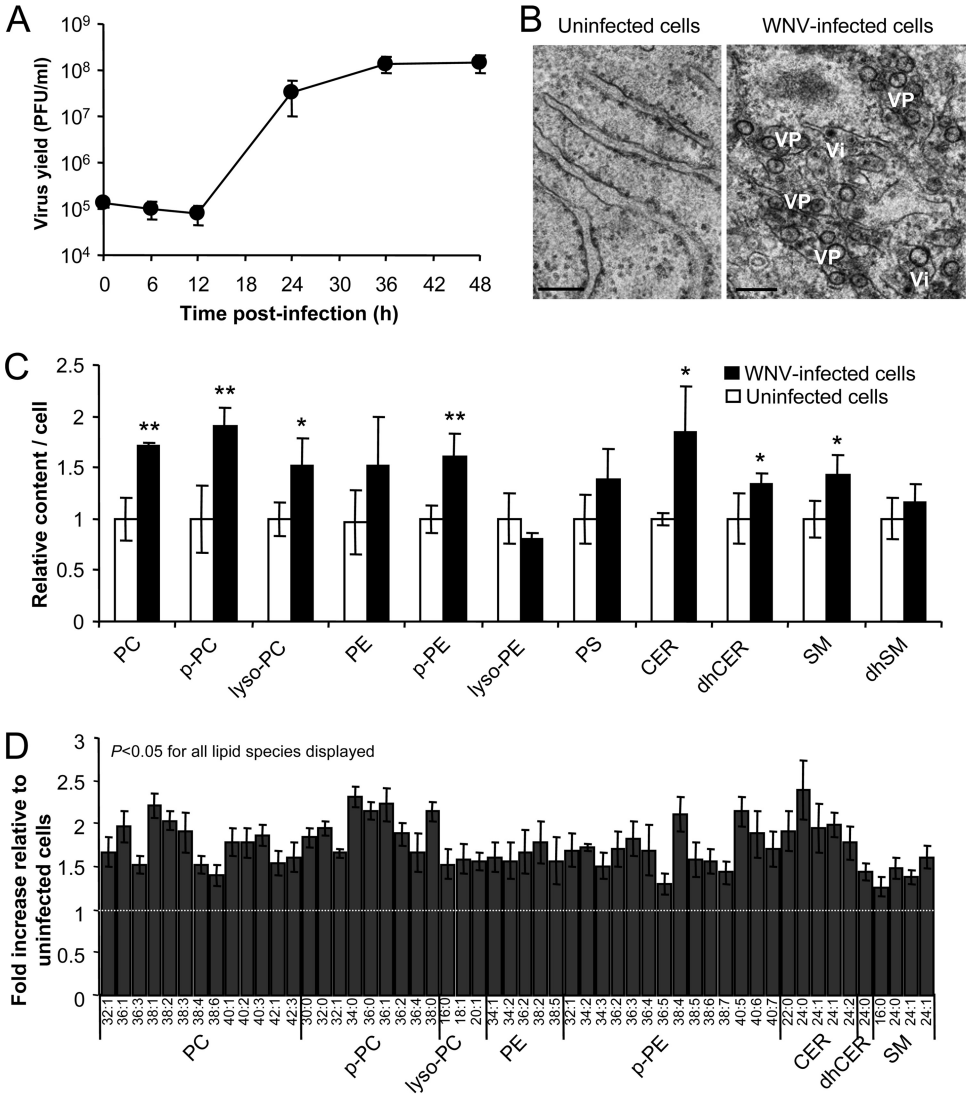

FIG 1 WNV infection alters lipid metabolism. (A) Time course analysis of WNV infection. HeLa cells were infected with WNV (MOI, 50 PFU/cell), and the amount of infectious virus released into the culture medium was determined by plaque assay at different times p.i. (B) Intracellular membrane rearrangements in WNV-infected HeLa cells observed by transmission electron microscopy. A micrograph showing the ER from uninfected cells is included for comparison. Characteristic features of flavivirus-infected cells, vesicle packets (VPs) and virions (Vi), were observed at 24 h p.i. (MOI, 50 PFU/cell). Bars, 200 nm. (C) Relative amount of different classes of GPLs and SLs in HeLa cells infected or not infected with WNV (MOI, 50 PFU/cell) determined by mass spectrometry at 24 h p.i. Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$. (D) Fold differences in individual lipid species significantly increased in infected cells analyzed as described in the legend to panel C. Dashed line, mean value for each lipid in uninfected cells. Data are presented as the mean ± SD.

from the ER in a manner parallel to that observed for infectious virus (6).

Hence, the lipid composition of the envelope from sucrose gradient-purified WNV and RSPs was analyzed by mass spectrometry and compared to that of total cellular membranes of HeLa cells (Fig. 4A) following an approach similar to that described for other viruses (7–10). The most abundant GPL in virions, RSPs, and total cellular membranes was PC, although both RSPs and virions displayed significantly reduced contents of PC relative to those of the total cellular membranes (about 0.57-fold for both RSPs and virions). Among the SLs, a significant enrichment in SM was noticed in the envelope of RSPs and virions compared to the amount in total cellular membranes (about 3.9- and 2.5-fold, respectively). These analyses also revealed that the lipid composition of both virions and RSPs was very similar for all lipid classes analyzed, except for p-PC, which appeared to be enriched in virions compared with the amounts in both RSPs and total cellular membranes. This could be related to the differences in the sizes between virions (50 nm) and RSPs (30 nm), since vesicles formed with p-PC are larger than those formed with PC (48).

Of the 162 lipid species identified in total cellular membranes, about 95% and 93% were also detected in RSPs ($n = 155$) and virions ($n = 152$), respectively (see Data Set S1 in the supplemental material). Significant differences in the proportion of lipid species between RSPs and virions were restricted to 3 lipid species (lyso-PC, 18:2; CER, 18:0; dhSM, 24:0; these represented only about 2% of the total species identified) (see Data Set S1 in the supplemental material), supporting the suggestion that the lipid

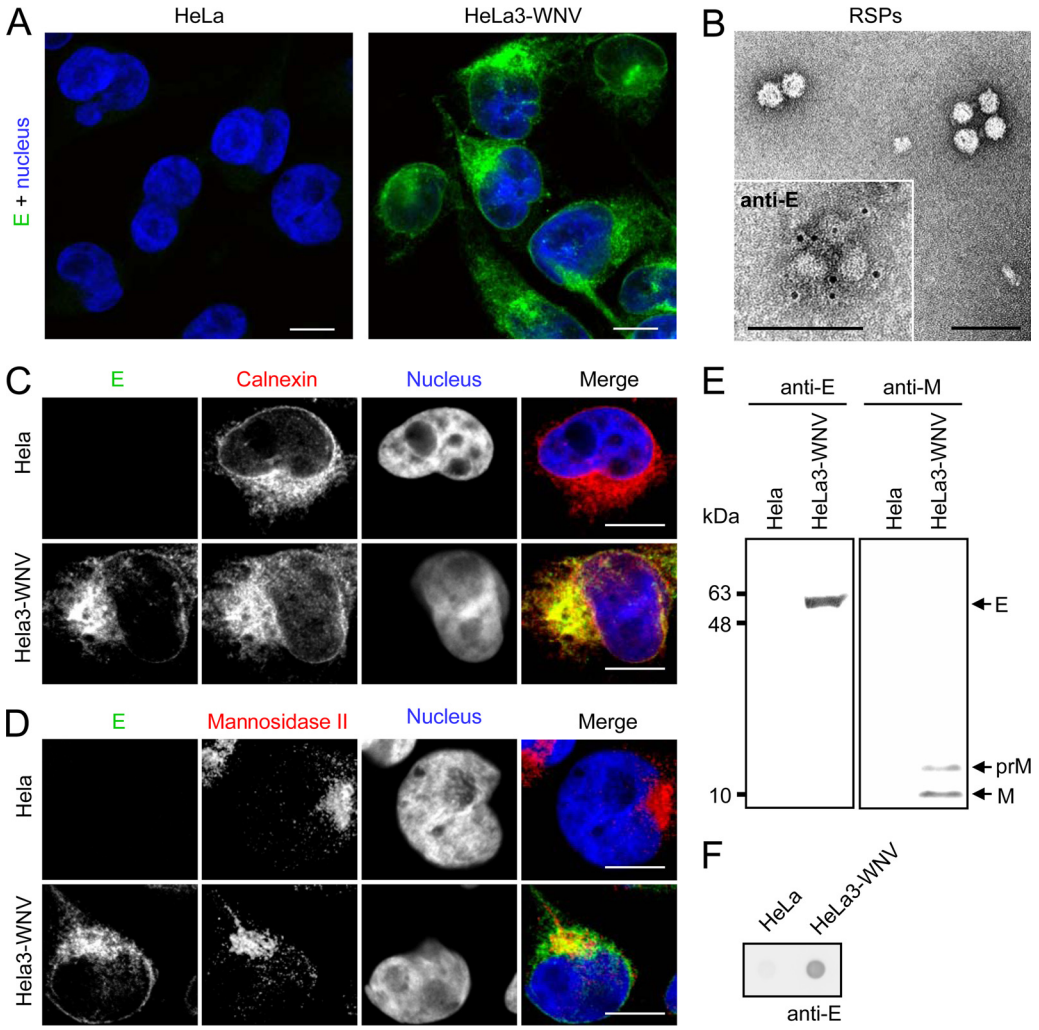

FIG 2 WNV RSP as a noninfectious system to study flavivirus biogenesis. (A) Immunofluorescence analysis of the expression of the WNV E glycoprotein in HeLa3-WNV cells. HeLa or HeLa3-WNV cells were subjected to immunofluorescence analysis using a monoclonal antibody directed against the E glycoprotein (green) and observed by confocal microscopy. Nuclei were stained with ToPro3 (blue). Bars, 10 μm. (B) RSPs produced by HeLa3-WNV cells. The RSPs released into the culture medium were purified by sucrose gradient centrifugation and observed by negative staining and transmission electron microscopy. (Inset) Immunogold staining of RSPs using a monoclonal antibody against the E protein. RSPs were detected using protein A coupled to 5-nm colloidal gold. Bars, 100 nm. (C and D) Localization of the WNV E glycoprotein at the ER (C) and the Golgi complex (D) of HeLa3-WNV cells. Immunofluorescence was performed using a monoclonal antibody directed against the E glycoprotein (green) in combination with rabbit polyclonal antibodies against calnexin (red) to stain the ER or mannosidase II (red) to stain the Golgi complex. Nuclei were stained with ToPro3 (blue). HeLa cells not expressing viral proteins were included as negative control. Bars, 10 μm. (E) Western blot analysis of RSPs purified through a sucrose cushion from the culture medium of HeLa3-WNV cells. Cell culture medium from control HeLa cells was processed and analyzed in parallel as a negative control. Western blotting was performed using anti-E or anti-M antibodies. (F) Enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein of culture supernatants from HeLa3-WNV cells. Culture medium from HeLa cells not expressing viral glycoproteins was included as a negative control.

envelopes of RSPs and virions share multiple common features. Compared to the lipid species in total cellular membranes, virions and RSPs displayed significantly altered proportions of 30 (19%) and 36 (24%) lipid species, respectively, with 15 of them being significantly altered in both virions and RSPs (Fig. 4B). Among these common species altered, 5 corresponded to PE or its derivative, p-PE (4 were enriched [PE, 32:1; p-PE, 32:2; p-PE, 34:3; p-PE, 36:3] and 1 was reduced [PE, 36:4]), and 6 corresponded to PC (34:1, 34:3, 36:2, 38:5, 38:6, and 44:3, all of which were reduced). Regarding SLs, the enrichment of dhCER (24:0) and three SM species (16:0, 24:0, and 24:2) was noticed. When lipids enriched in both RSPs and virions (Fig. 4B) were compared with those increased in infected cells (Fig. 1D), only 5 matches were found (p-PE, 34:3; p-PE, 36:3; dhCER, 24:0; SM, 24:0; SM, 24:1). Interestingly, three of these species were SLs, suggesting that the enrichment of these molecular species in infected cells could be related to an increasing demand for these SLs for the assembly of the viral envelope.

Involvement of SM and CER in flavivirus biogenesis. The SLs are derived from sphingosine, a long-chain amino alcohol, which is acylated with a long-chain fatty acid to give CER, which in turn is the central core of SM. These lipids are important components for membrane organization and shape, playing key roles in multiple cellular processes (49). Our analysis showed that different

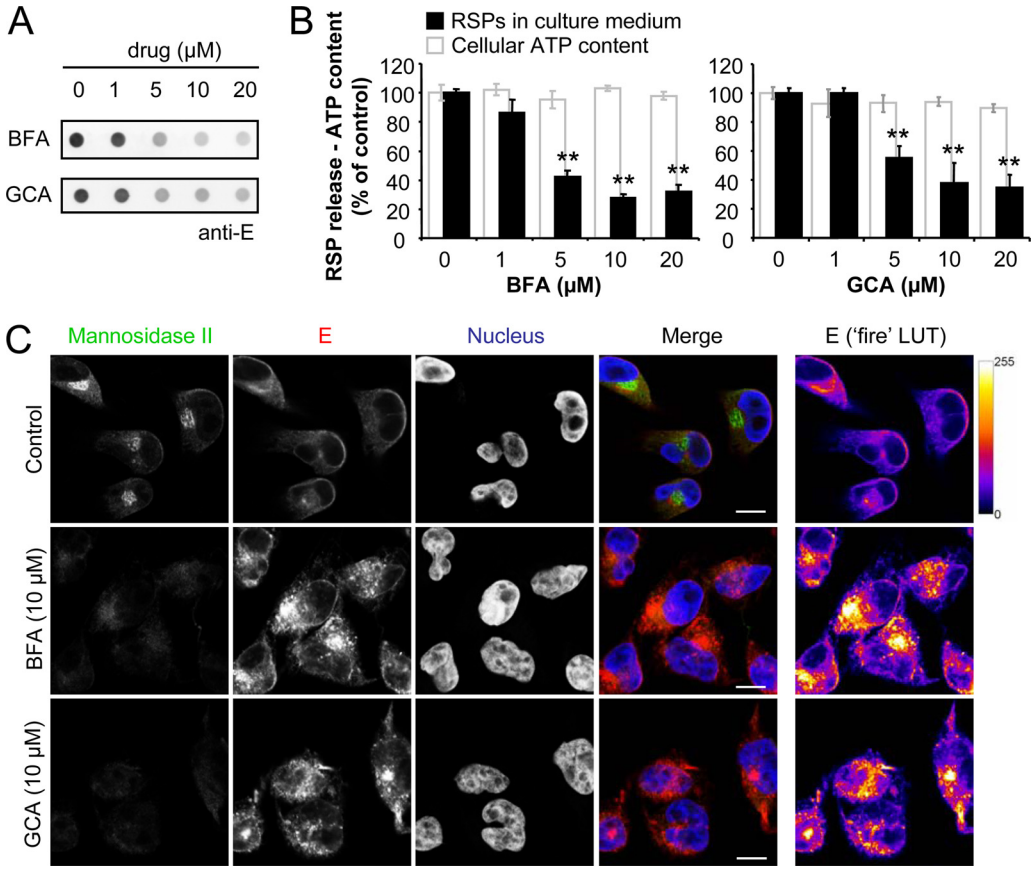

FIG 3 Treatment with BFA or GCA impairs the secretion of RSPs into the culture medium. (A) RSP release into the culture medium by HeLa3-WNV cells treated (4 h) with the drugs was analyzed by enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein. (B) Quantification of the amount of RSPs released into the culture medium by treated HeLa3-WNV cells from panel A. For each drug concentration, the cellular ATP content is indicated as an indicator of cell viability upon drug treatment. (C) Localization of the WNV E glycoprotein in infected HeLa cells treated with Golgi complex-disrupting agents. HeLa3-WNV cells were treated with 10 μM BFA or GCA for 4 h. Immunofluorescence was performed using a monoclonal antibody directed against the WNV E glycoprotein (red) in combination with a rabbit polyclonal antibody against mannosidase II (green) to stain the Golgi complex. Nuclei were stained with ToPro3 (blue). The panels in the rightmost column display E glycoprotein staining with false coloring from dark purple to bright yellow by use of the fire lookup table (LUT) scheme to highlight differences in the intensities of the signals. Bars, 10 μm.

SLs (CER, dhCER, and SM) were increased in infected cells (Fig. 1C); this was particularly the case for SM, which was found to be enriched in the lipid envelope of both RSPs and virions (Fig. 4A). The hydrolytic removal of the phosphocholine moiety of SM by sphingomyelinases (SMases) to render CER has been associated with the induction of membrane curvature in SL-enriched membrane domains (50, 51). In fact, compounds that inhibit the activity of the neutral SMase 2 (nSMase2; also named sphingomyelin phosphodiesterase 3 [*SMPD3*]) can inhibit budding processes (18). Since these observations are compatible with the potential roles of SLs during WNV infection, i.e., in virion budding and/or assembly, we first addressed the effect of nSMase inhibition in HeLa3-WNV cells, used as a model system to study flavivirus biogenesis in the absence of virus replication. To this end, we selected three structurally unrelated nSMase inhibitors (18): GW4869, a noncompetitive inhibitor of nSMase (34); spiroepoxide, a selective irreversible inhibitor of nSMase (52); and glutathione, a cellular regulator of nSMase activity (53). Treatment of these cells with either of these inhibitors significantly reduced the amount of RSPs released into the culture medium without exerting noticeable toxic effects on the cells (Fig. 5A to C). GW4869 was the drug that produced significant inhibition at a lower concentration, so this compound was selected for further experiments involving virus infections (see below). As a complementary approach, the effect of nSMase2 depletion using RNA interference was also analyzed. Silencing of the expression of nSMase2 by transfection with siRNA, which was verified by Western blotting (Fig. 5D), significantly inhibited the release of RSPs into the culture medium (Fig. 5D and E). When the E-protein content in siRNA-transfected cells was analyzed by Western blotting (Fig. 5D) or immunofluorescence (Fig. 5F), no reduction was observed in cells transfected with siRNA against nSMase2. These results point to an accumulation of viral proteins inside cells depleted of nSMase2, which is consistent with the reduction in the release of RSPs from cells depleted of nSMase2 that was observed. The extent of the inhibition of RSP release in cells depleted of nSMase2 by siRNA (Fig. 5E) was comparable to that exerted by treatment with nSMase inhibitors (Fig. 5A to C), further supporting the requirement for nSMase function during the biogenesis of flaviviruses.

Interference with nSMase function reduces flavivirus release from infected cells. The effect of the nSMase inhibitor GW4869 on WNV infection was assayed in two mammalian cell lines (HeLa

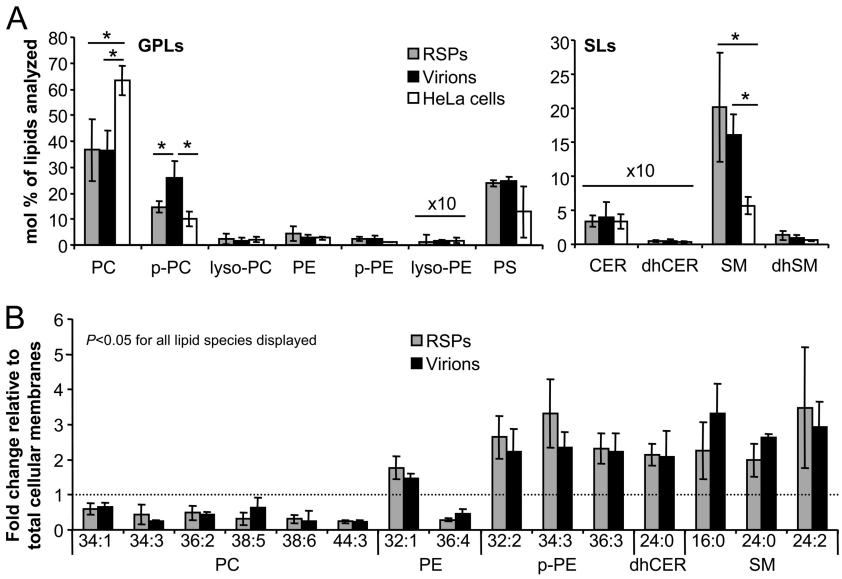

FIG 4 Lipid composition of WNV RSPs and virions. (A) GPL and SL compositions of WNV RSPs and virions. The content of individual lipid classes of total HeLa cells and WNV RSPs or virions was determined by summing the absolute abundances of all species identified. Values are standardized to the mole percentage of all membrane lipids detected in the sample. Values multiplied by 10 for visibility are indicated. (B) Individual lipid species whose levels were significantly altered in virions or RSPs in comparison to their levels in total cellular membranes. Data are presented as the mean ± SD. Statistically significant differences are indicated: *, $P < 0.05$.

and Vero) and one mosquito cell line (C6/36) (Fig. 6A to C). Treatment with GW4869 at concentrations of ≥5 μM significantly inhibited the release of infectious WNV in these three cell lines, confirming the requirement for nSMase for the production of WNV in both mammalian and insect cells. The effect of this inhibitor on infection with Usutu virus (USUV), a related emerging flavivirus, was also tested in HeLa cells (Fig. 6D). In these experiments, inhibition of USUV similar to that of WNV at drug concentrations of ≥5 μM was observed. To analyze whether the requirement for nSMase function for virus production was shared by other unrelated arboviruses, the effect of GW4869 on infection with the alphavirus Sindbis virus (SINV) was analyzed (Fig. 6E). Interestingly, the release of SINV infectious particles from HeLa cells was not inhibited but was significantly increased by treatment with GW4869. This enhancing effect on virus titer was similar to that previously reported when SINV infection was performed in cells exhibiting an altered SL content induced by depletion of acid SMase, which was associated with an increase in virion infectivity (54). We next addressed the effect of depletion of nSMase2 on infection with these three viruses by transfection with specific siRNA (Fig. 6F). Silencing of nSMase2 expression significantly inhibited the production of both WNV and USUV to an extent comparable to that produced by treatment with GW4869. Conversely, a significant increase in SINV titer was found in cells treated with siRNA against nSMase2. Therefore, these observations confirm the results obtained with GW4869 and highlight the involvement of nSMase function during flavivirus infection.

Inhibition of nSMase activity reduces WNV-induced membrane budding. The mechanism behind the reduction of WNV growth upon nSMase inhibition using GW4869 was further studied. The amount of viral particles released into the culture medium showed a significant reduction, as determined by an enzyme-linked immunodot assay analyzing the levels of E glycoprotein secreted in cells treated with GW4869 (Fig. 7A). This result was consistent with the decrease in RSP and infectious virus release observed (Fig. 5A and 6A). Analysis of infected cells treated with GW4869 by immunofluorescence and confocal microscopy showed an accumulation of E glycoprotein colocalized with the ER marker calnexin (Fig. 7B). To rule out the possibility that the effect of GW4869 on the biogenesis of WNV occurred in a step after virus assembly or maturation, the amount of cell-associated infectious virus or infectious virus released into the medium was determined for cells treated with GW4869 (Fig. 7C). Whereas treatment with GW4869 significantly reduced the amount of infectious WNV released, it did not induce an accumulation of cell-associated infectious virus. When the amounts of cell-associated viral RNA and viral RNA released into the medium were analyzed (Fig. 7D), a significant reduction of viral RNA levels in the culture medium of cells treated with GW4869 was observed. Conversely, a significant accumulation of cell-associated viral RNA was noticed. Overall, these results are compatible with a reduction in the amount of infectious virus released with a concomitant accumulation of viral RNA but not the amount of infectious particles inside GW4869-treated cells, a result which suggests that inhibition of nSMase function alters a process of flavivirus biogenesis before the assembly and/or maturation of infectious particles. Interestingly, the extent of the inhibition of PFU release (about 60%; Fig. 7C) was higher than the extent of the inhibition of total particle or viral RNA release (about 40%; Fig. 7A and D). This could suggest that GW4869 not only decreases the amount of released viral particles but also elevates the proportion of secreted noninfectious particles.

As the inhibition of nSMase function can interfere with intracellular budding processes, the effect of GW4869 on virus-mediated budding was analyzed. To this end, infected cells were treated with GW4869 and analyzed by transmission electron microscopy (Fig. 7E). Cells treated with the nSMase inhibitor displayed ultrastructural features associated with flavivirus infection, i.e., the formation of VPs

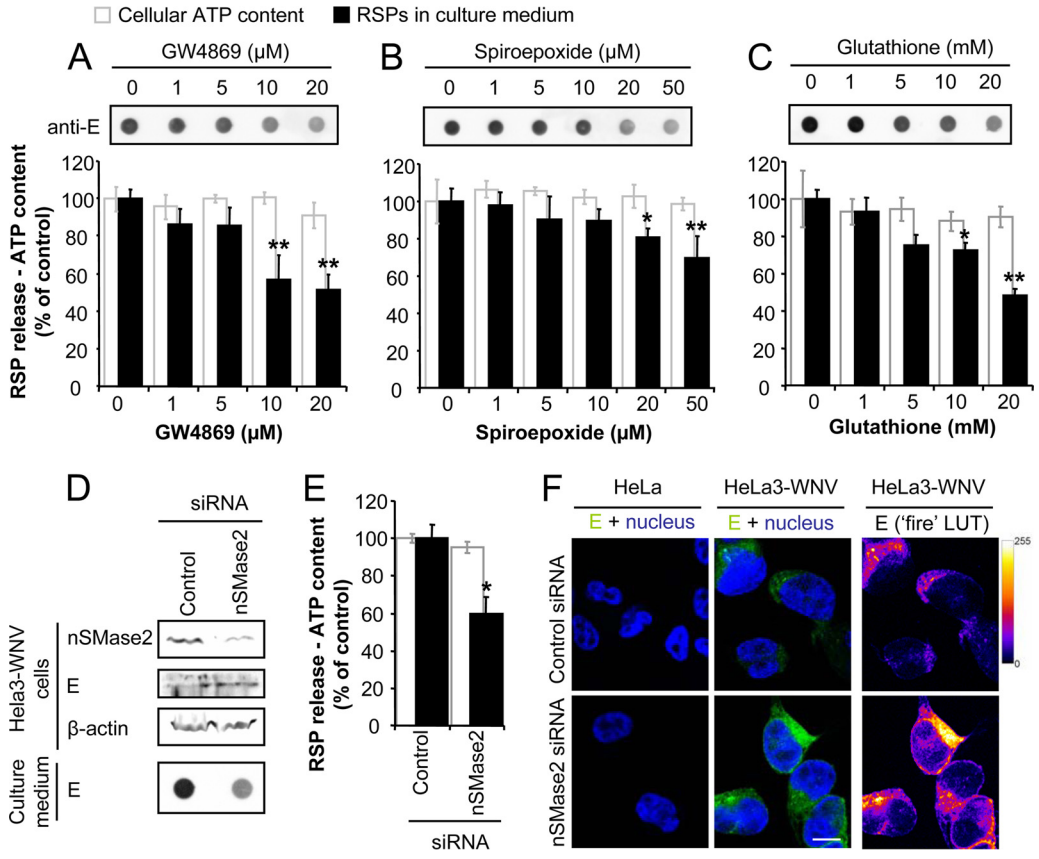

FIG 5 Impairment of nSMase function inhibits the release of RSPs. (A to C) Treatment with the nSMase inhibitor GW4869 (A), spiroepoxide (B), or glutathione (C) impairs the release of RSPs by HeLa3-WNV cells. The content of RSPs in the culture medium of cells treated (for 4 h) with the drugs was determined by enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein. The amount of RSPs determined by enzyme-linked immunodot assay was quantified, and the cellular ATP content for each drug concentration is shown in the graphs. (D) Depletion of nSMase2 by RNA interference. HeLa3-WNV cells were transfected with universal negative-control siRNA or with siRNA against nSMase2 for 48 h, and the amount of nSMase2, E glycoprotein, and β-actin in cell lysates was analyzed by Western blotting using specific antibodies. RSP release into the culture medium after 4 h of incubation in cells transfected for 48 h with siRNA was analyzed by enzyme-linked immunodot assay using an anti-E antibody. (E) Quantification of the amount of RSPs released into the culture medium by HeLa3-WNV cells transfected with siRNA control or against nSMase2 from panel D. The cellular ATP content for each drug concentration is shown as an indicator of cell viability upon drug treatment. (F) Immunofluorescence analysis of the expression of the WNV E glycoprotein in HeLa3-WNV cells transfected with siRNAs. HeLa or HeLa3-WNV cells transfected as described in the legend to panel D were subjected to immunofluorescence analysis using an antibody directed against E glycoprotein (green) and observed by confocal microscopy. Nuclei were stained with ToPro3 (blue). The panels on the right display E glycoprotein staining with false coloring from dark purple to bright yellow by use of the fire lookup table (LUT) to highlight differences in the intensity of the signal. Bars, 10 μm. Data are presented as the mean ± SD. Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

and the presence of electron-dense virions. VPs are produced by budding into the ER, and the abundance of these structures can be quantified by transmission electron microscopy in flavivirus-infected cells (55). According to this view, these structures were analyzed to directly evaluate the effect of nSMase inhibition on viral budding. The mean diameter of the virus-induced vesicles detected inside the VPs (about 80 nm) in control and GW4869-treated cells was indistinguishable (Fig. 7F). However, when the number of vesicles inside VPs was scored, a significant reduction in the number of vesicles inside each VP in GW4869-treated cells relative to the number in control infected cells was noted (mean numbers of vesicles per VP, 1.9 ± 0.2 and 5.8 ± 0.6, respectively; Fig. 7G), supporting the suggestion that viral budding into the ER was reduced upon inhibition of nSMase function.

DISCUSSION

Our results illustrate the alteration of the content of a wide variety of lipid metabolites in WNV-infected cells. The increase in the cellular content of PC, lysophospholipids (lyso-PC), SM, and CER in HeLa cells infected with WNV is consistent with previous results with mosquito cells infected with DENV (26). Our analysis also revealed the enrichment of p-PC, p-PE, and dhCER in WNV-infected cells. Apart from PC, which is a cylindrical lipid, the rest of the lipids whose levels were found to be increased in WNV-infected cells were conical. The increase in the levels of these conical lipids could be related to their roles in the establishment and maintenance of the cellular membrane curvature necessary for proper viral replication complex assembly, as well as for virion envelopment. The enrichment of unsaturated PC species in DENV-infected cells has been suggested to be associated with the development of more fluidic membranes (26). Supporting this idea, all PC species whose levels were increased in WNV-infected cells were unsaturated. Likewise, the observed enrichment of SLs (CER, dhCER, and SM) in WNV-infected cells is also compatible with the require-

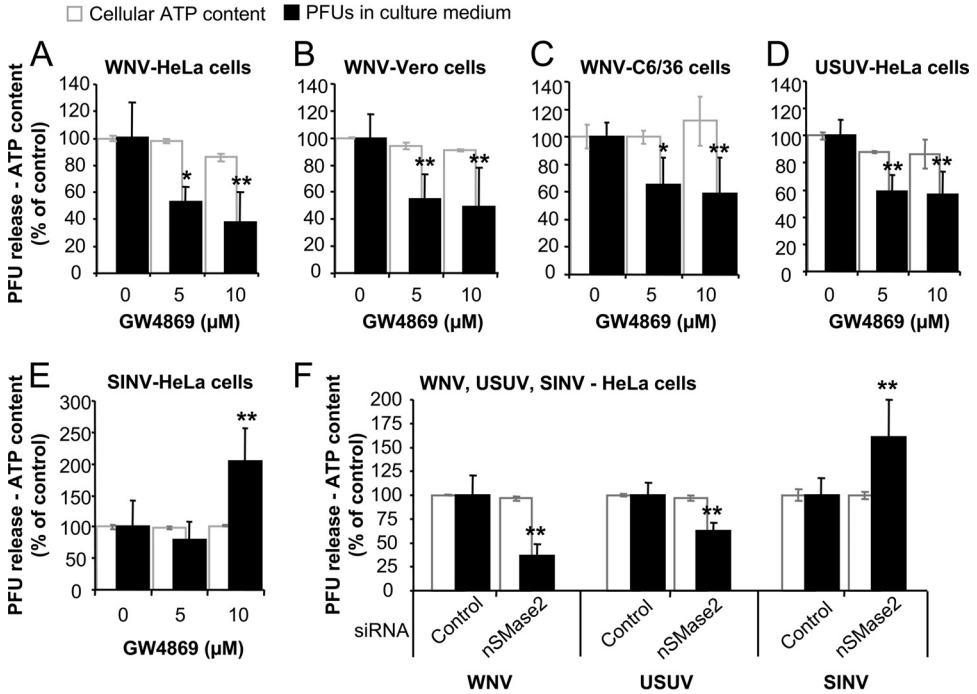

FIG 6 Impairment of nSMase function inhibits the release of WNV. (A to C) Treatment with the nSMase inhibitor GW4869 reduces WNV production in mammalian and insect cells. HeLa (A), Vero (B), or C6/36 (C) cells were infected with WNV (MOI, 1 PFU/cell) and treated with the indicated drug, and the amount of infectious virus released into the culture medium was determined by plaque assay at 24 h p.i. The cellular ATP content of uninfected cells treated with the different concentrations of the drug is indicated in all graphs as an estimate of cell viability upon drug treatment. (D) Treatment with GW4869 reduces USUV production. HeLa cells were infected with USUV (MOI, 1 PFU/cell), and the amount of infectious virus released was determined as described in the legends to panels A to C. (E) Treatment with GW4869 increases SINV production. HeLa cells were infected with SINV (MOI, 1 PFU/cell), and the amount of infectious virus released into the culture medium was determined by plaque assay (8 h p.i.). (F) Effect of nSMase2 depletion by siRNA on WNV, USUV, and SINV infection. HeLa cells were transfected with a universal negative siRNA control or with siRNA against nSMase2 for 48 h and infected with WNV, USUV, or SINV, and the amount of virus released into the culture medium was analyzed as described in the legend to panels A to C. Data are presented as the mean ± SD. Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

ment for these molecules in the membranes where virus replication and assembly take place. According to this hypothesis, it has been documented that DENV and hepatitis C virus replication complexes are enriched in SLs (17, 26).

To identify the potential roles of the lipid classes analyzed in the biogenesis of WNV particles, the proportion of each lipid in both purified virions and RSPs was analyzed. All lipid classes analyzed, including PS, which has been shown to play a functional role during flavivirus entry, were detected in the envelopes of WNV and RSPs (11). The high degree of similarity between the lipid composition of RSPs and virions, along with their parallel mechanisms of formation, reinforces the view that RSPs are a valuable tool for the study of lipid involvement during the biogenesis of flaviviruses.

We next addressed the question of whether the lipid composition of the WNV envelope was similar to that of total host cell membranes or if it reflected that of a particular membrane domain, following an approach similar to the approaches used for the characterization of other viral envelopes (7, 10). This analysis indicated that the envelopes of both RSPs and virions were enriched in SM and exhibited a reduction in the content of PC compared to that of total cellular membranes. Similar features, such as an increase in the SM (or other SL) content and a depletion of PC, have also been observed in the lipid envelopes of human immunodeficiency virus type 1 (HIV-1) and influenza virus (7, 9). On the contrary, the rhabdovirus vesicular stomatitis virus and the alphavirus Semliki Forest virus exert little lipid selection when they acquire their envelopes (8). The characteristics of the WNV envelope also differed from those of the hepatitis C virus envelope, which more closely resembles the composition of very low- and low-density lipoproteins (10). Enrichment in SLs and a reduction of PC levels are characteristics of liquid ordered membrane microdomains or membrane rafts (7, 9, 18, 56). Since membrane raft-like microdomains, including those located in the ER (57), are usually cholesterol enriched (7, 9, 18, 56), our findings could be connected to the role(s) of membrane microdomains enriched in cholesterol in flavivirus infections (28, 31, 58–60). SL-enriched membrane microdomains have functional properties directly linked to their lipid composition, such as coalescence and the promotion of budding processes during virus assembly (7, 9) or exosome biogenesis (18). Indeed, flavivirus assembly by invagination of ER membrane shares topological features with the formation of exosomes, since both processes mainly consist of budding outward from the cell cytoplasm. Supporting this hypothesis, the parallelism between viral budding processes and exosome biogenesis has already been documented (61, 62). An important point that must also be considered in these analyses is that in the RSP system, HeLa3-WNV cells express viral prM and E proteins in the absence of any other viral protein. Since viral nonstructural proteins (such as NS4A) induce membrane proliferation (63, 64),

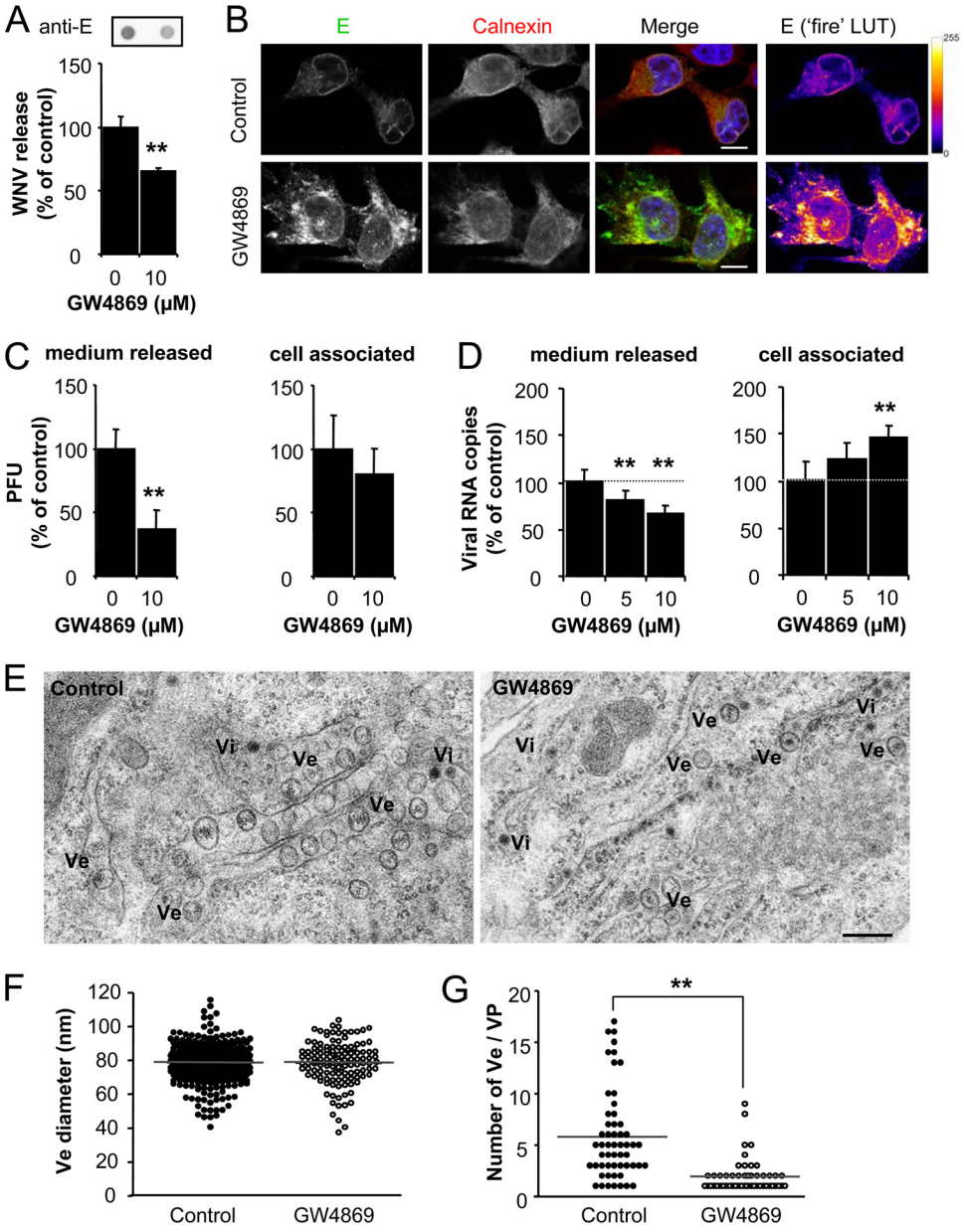

FIG 7 Inhibition of nSMase function reduces WNV-induced budding. (A) Treatment with the nSMase inhibitor GW4869 reduces WNV release. HeLa cells were infected with WNV (MOI, 1 PFU/cell) and treated with the drug, and the amount of virus particles released into the culture medium was estimated by an enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein at 24 h p.i. (B) Localization of the WNV E glycoprotein in HeLa cells infected with WNV (MOI, 1 PFU/cell) and treated with 10 μM GW4869. Immunofluorescence was performed using a monoclonal antibody directed against the E glycoprotein (green) in combination with a rabbit polyclonal antibody against calnexin (red) to stain the ER at 24 h p.i. Nuclei were stained with ToPro3 (blue). The panels in the rightmost column display E glycoprotein staining with false coloring from dark purple to bright yellow by use of the fire lookup table (LUT) scheme to highlight differences in the intensities of the signals. Bars, 10 μm. (C) Impairment of nSMase function with GW4869 does not increase the amount of cell-associated WNV. HeLa cells were infected with WNV (MOI, 1 PFU/cell), and the amount of virus either released or cell associated was determined by plaque assay at 24 h p.i. (D) The amount of viral RNA released into the culture medium or cell associated was determined by quantitative RT-PCR in cells infected and treated as described in the legend to panel C. (E) Representative electron micrographs at 24 h p.i. of cells infected with WNV (MOI, 10 PFU/cell) and treated or not treated (control) with 10 μM GW4869 showing the formation of vesicle packets (VPs) containing virus-induced vesicles (Ve) and electron-dense virions (Vi). Bar, 200 nm. (F) Diameter of virus-induced vesicles detected in the VPs of cells infected and treated or not treated with 10 μM GW4869. Each point represents an individual virus-induced vesicle. Solid lines denote the mean vesicle diameter. (G) Number of virus-induced vesicles per VP detected in the VPs of cells treated or not treated with 10 μM GW4869. Each point represents the number of virus-induced vesicles detected in an individual VP. Solid lines denote the mean number of virus-induced vesicles per VP. Unless specified otherwise, data are presented as the mean ± SD. Statistically significant differences are indicated: **, $P < 0.005$.

their expression is expected to modify cellular lipids in infected cells. In fact, modification of cellular lipids upon infection with WNV was confirmed by analysis of the lipids in WNV-infected cells. Therefore, it is foreseeable that the lipid components from WNV-infected cells can differ from those of RSP-producing cells, which may explain some of the minor differences between RSPs and virions found in the analysis, such as the amount of p-PC or variations in the specific lipid species found between RSPs and

virions. However, the major features observed (a reduction of PC and enrichment in SM) were common between the two systems, supporting the use of RSPs as a model to study flavivirus biogenesis.

The hydrolysis of SM by nSMases converts SM into CER, a compound with a smaller head size (49). CER induces an asymmetric membrane tension and segregates into highly ordered domains triggering modifications of membrane shape (65). These properties have been associated with the promotion of membrane bending and budding processes both on model membranes (50) and in living cells (18, 51, 66, 67). In this scenario, pharmacological inhibition of nSMase activity, which catalyzes the hydrolysis of SM to CER, impaired the release of RSPs into the culture medium and reduced the level of production of infectious particles of WNV and the related flavivirus USUV, which parallel the effects on the production of certain types of exosomes that have been documented (18, 66, 67). Supporting this observation, depletion of nSMase2 by RNA interference inhibited the release of RSPs and the production of WNV and USUV. On the contrary, an increase in the titer of another arbovirus (SINV) was observed upon inhibition of nSMase function, therefore indicating that the requirement for the conversion of SM to CER during the biogenesis of flavivirus is not shared by this alphavirus. Consistent with these results, lipid recruitment during alphavirus envelopment has been proposed to be a process with a low level of selection (8), in contrast to the enrichment of SM and the reduction in the level of PC described here for the flavivirus envelope. Alphavirus biogenesis is dependent on the functionality of the secretory pathway, since viral envelope glycoproteins must traffic from the ER through the Golgi complex to the plasma membrane for viral budding (68). Thus, the lack of inhibition of SINV release upon pharmacological inhibition of nSMase2 or siRNA-mediated depletion of this enzyme shows the functionality of the secretory pathway in cells treated with GW4869 or siRNA against nSMAse2. This observation further supports the specific involvement of nSMase2 in the biogenesis of flavivirus. Although other nSMases are expected to be functional in cells depleted of nSMase2 by siRNA, the degree of inhibition (of RSPs and virion release) achieved with specific siRNA against nSMase2 was similar to that observed using the nSMase inhibitor GW4869, thus reinforcing the idea that nSMase2 is the main nSMase involved in flavivirus biogenesis.

When the effect of nSMase inhibition on virus biogenesis was analyzed by several approaches, a reduction of the amount of virus-induced vesicles inside VPs in cells treated with an inhibitor of nSMase function was noticed. Since these vesicles are associated with flavivirus replication and virion envelopment (5, 43, 55), this observation supports the role of SM conversion to CER in virus-induced budding. The diameter of the vesicles found in cells treated with the nSMase inhibitor was similar to that of the vesicles observed in untreated cells. A possible explanation for this phenomenon could be that vesicle components others than lipids (presumably, viral proteins) also contribute to vesicle formation, although the inhibition of nSMase activity makes this assembly less efficient. Accordingly, an important role for viral glycoproteins in the induction and maintenance of membrane curvature in flavivirus virions has been proposed (69). Since budding mechanisms are usually driven by a regulated combination of proteins and lipids (70), our results indicate that, indeed, the lipid composition of the flavivirus envelope also contributes to this process.

Interestingly, the dhCER content was found to be increased in WNV-infected cells, as it was one of the molecular species enriched in the viral envelope (dhCER, 24:0). This metabolite is an intermediate in the SL biosynthetic pathway (49), and the presence of increased levels of dhCER points to the upregulation of the *de novo* synthesis of SLs within WNV-infected cells, as reported in other virus models (17, 26). On the other hand, the involvement of nSMase in WNV and USUV biogenesis indicates that the production of CER by the hydrolysis of SM is also required during infection with these pathogens, providing evidence of the functionality of these two pathways for CER production in flavivirus-infected cells. However, one must be cautious with the interpretation of the reason for the increase in CER levels in WNV-infected cells, since this can be due to different factors, such as the induction of ER stress (71) mediated by the activation of the unfolded protein response in flavivirus-infected cells (72).

In summary, our results provide additional evidence of the complex degree of manipulation of host cell lipid metabolism by WNV. These results also unveil a connection between SL metabolism and flavivirus biogenesis. The findings presented here could contribute to the further development of lipid-based antiviral strategies to combat these pathogens.

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Artículo II

Merino-Ramos T, Vázquez-Calvo Á, Casas J, Sobrino F, Saiz JC, Martín-Acebes MA. **"**Modification of the host cell lipid metabolism induced by hypolipidemic drugs targeting the acetyl coenzyme A carboxylase impairs West Nile virus replication". Antimicrob Agents Chemother. 2015 Oct 26;60(1):307-15. doi: 10.1128/AAC.01578-15. PMID: 26503654

En línea con nuestras investigaciones previas sobre la inhibición de los factores celulares del hospedador (Artículo I), continuamos manipulando los lípidos celulares en el contexto de la infección por flavivirus con el objetivo de adquirir un mayor entendimiento del requerimiento de éstos en el ciclo de vida viral. En este sentido, se sabe que los primeros pasos en la biogénesis lipídica implican la síntesis y elongación de ácidos grasos, la cual es la base para la síntesis de lípidos más complejos. Por tanto, en el **Artículo II**, analizamos el efecto de inhibidores de la enzima acetil-Coenzima A Carboxilasa (ACC) sobre la infección por VNO, ya que la ACC cataliza la carboxilación de la acetil-CoA a malonil-CoA, uno de los primeros pasos en la síntesis lipídica. El **Artículo II** muestra que los inhibidores de ACC alteran la composición lipídica celular y reducen los niveles de infección por VNO y virus de Usutu en cultivo celular, señalando a la ACC como una posible diana terapéutica para el desarrollo de antivirales contra múltiples virus dentro del mismo género o familia.

Durante el desarrollo de esta investigación llevé a cabo la mayoría del trabajo experimental desarrollado y contribuí al diseño de una parte significativa de los experimentos. Realicé las infecciones de células (Vero, HeLa y C6/36) con virus (VNO y USUTU) y su tratamiento con concentraciones crecientes de distintos fármacos (5-(tetradecyloxy)-2-furoic acid, TOFA, y 3,3,14,14-tetramethylhexadecanedioic acid, MEDICA 16), determinando la toxicidad de los mismos en las distintas células utilizadas y titulando las progenies víricas obtenidas. Obtuve y cuantifiqué el ARN de doble cadena mediante inmunofluorescencia en células infectadas, tratadas o no con fármaco. Así mismo, obtuve el ARN viral y lo analicé por RT-PCR cauntitativa. Finalmente llevé a cabo parte del análisis de los datos y contribuí a la escritura del artículo detallando los resultados obtenidos

Article II

Merino-Ramos T, Vázquez-Calvo Á, Casas J, Sobrino F, Saiz JC, Martín-Acebes MA. **"**Modification of the host cell lipid metabolism induced by hypolipidemic drugs targeting the acetyl coenzyme A carboxylase impairs West Nile virus replication". Antimicrob Agents Chemother. 2015 Oct 26;60(1):307-15. doi: 10.1128/AAC.01578-15. PMID: 26503654

In line with our previous investigation in host cell factors inhibition (Article I), we continued manipulating cellular lipids in the context of flavivirus infection with the aim of better understanding their requirement in the virus life cycle. In this regard, it is known that the first steps of lipid biogenesis involve the synthesis and elongation of fatty acids, which provide the cornerstone for the synthesis of more complex lipids. Therefore, in **Article II** the effect of inhibitors of the enzyme acetyl-Coenzyme A carboxylase (ACC) on the infection by WNV was analyzed, as ACC catalyze the carboxylation of acetyl-CoA to malonyl-CoA, one of the first steps of lipid synthesis. **Article II** shows that ACC inhibitors alter the cellular lipid composition and reduce the level of WNV and USUV infection in cultured cells, pointing to the ACC as a potential druggable cellular target suitable for antiviral development against multiple viruses within the same genus or family.

Along the development of this research, I carry out most of the experimental work, and I contributed to the experimental design. I made the cells infections (Vero, HeLa and C6/36) with viruses (WNV and USUV) and its treatment with increasing concentrations of different drugs (5-(tetradecyloxy)-2-furoic acid (TOFA) y 3,3,14,14-tetramethylhexadecanedioic acid (MEDICA 16). Previously, I determined drug toxicity in the same cell lines and afterwards, I made the viral titration. I obtained and quantified dsRNA intermediates by immunofluorescence in infected cells treated or not with drugs. Likewise, I performed viral RNA extractions and analyzed them by quantitative RT-PCR. Finally, I did a part of the data analysis and contributed to the article writing, detailing the obtained results.

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Modification of the Host Cell Lipid Metabolism Induced by Hypolipidemic Drugs Targeting the Acetyl Coenzyme A Carboxylase Impairs West Nile Virus Replication

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West Nile virus (WNV) is a neurotropic flavivirus transmitted by the bite of mosquitoes that causes meningitis and encephalitis in humans, horses, and birds. Several studies have highlighted that flavivirus infection is highly dependent on cellular lipids for virus replication and infectious particle biogenesis. The first steps of lipid synthesis involve the carboxylation of acetyl coenzyme A (acetyl-CoA) to malonyl-CoA that is catalyzed by the acetyl-CoA carboxylase (ACC). This makes ACC a key enzyme of lipid synthesis that is currently being evaluated as a therapeutic target for different disorders, including cancers, obesity, diabetes, and viral infections. We have analyzed the effect of the ACC inhibitor 5-(tetradecyloxy)-2-furoic acid (TOFA) on infection by WNV. Lipidomic analysis of TOFA-treated cells confirmed that this drug reduced the cellular content of multiple lipids, including those directly implicated in the flavivirus life cycle (glycerophospholipids, sphingolipids, and cholesterol). Treatment with TOFA significantly inhibited the multiplication of WNV in a dose-dependent manner. Further analysis of the antiviral effect of this drug showed that the inhibitory effect was related to a reduction of viral replication. Furthermore, treatment with another ACC inhibitor, 3,3,14,14-tetramethylhexadecanedioic acid (MEDICA 16), also inhibited WNV infection. Interestingly, TOFA and MEDICA 16 also reduced the multiplication of Usutu virus (USUV), a WNV-related flavivirus. These results point to the ACC as a druggable cellular target suitable for antiviral development against WNV and other flaviviruses.

West Nile virus (WNV) is a mosquito-borne neurotropic flavivirus responsible for recurrent outbreaks of meningitis and encephalitis affecting humans, horses, and birds in Africa, Europe, Asia, Oceania, and America (1). A great effort has been devoted in the past several years to decipher the molecular biology of WNV and its interaction with the host immune system (2, 3). Nevertheless, no licensed vaccine or therapy for human use against this pathogen is yet available.

The flavivirus life cycle (including that of WNV) is intimately associated with host cell lipids. The replication of the viral genomic RNA and flavivirus nascent virion assembly take place in modified membranes from the endoplasmic reticulum (4–7). To build an adequate microenvironment to support viral replication and particle biogenesis, flaviviruses rearrange host cell lipid metabolism by promoting the synthesis and accumulation of specific cellular lipids (i.e., fatty acids, glycerophospholipids [GPLs], sphingolipids [SLs], and cholesterol) (8–15). This makes the pharmacological manipulation of cellular lipids an attractive antiviral strategy against WNV and related flaviviruses (13, 14, 16, 17).

The first steps of lipid biogenesis involve the synthesis and elongation of fatty acids, which provide the building blocks for the synthesis of more-complex lipids. Hence, fatty acid synthesis and elongation have become key targets for antiviral therapy (13, 18, 19). Regarding the flaviviruses, the pharmacological blockage of the fatty acid synthase FASN (which catalyzes the synthesis of palmitate from acetyl coenzyme A [acetyl-CoA] and malonyl-CoA into long-chain saturated fatty acids) reduced the viral replication (11, 13). The enzyme preceding FASN in the fatty acid biosynthetic route is the acetyl-CoA carboxylase (ACC), which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. Due to its rate-limiting role in fatty acid synthesis, ACC is currently a target of increasing interest within the pharmacological industry (20, 21). However, to our knowledge, the involvement of ACC in the replication of WNV, or other related flaviviruses, has not yet been evaluated.

In this work, we have shown that ACC inhibitors alter the cellular lipid composition and reduce the levels of WNV infection in cultured cells. Furthermore, infection by Usutu virus (USUV; a related emerging flavivirus [22]) was also inhibited by the drugs used. Our results point to ACC as a potential druggable antiviral target against WNV and related flaviviruses.

MATERIALS AND METHODS

Cells, viruses, infections, and virus titrations. All infectious virus manipulations were performed in biosafety level 3 (BSL-3) facilities. Vero, HeLa, and Neuro2a (N2a) cell lines were cultured as described previously (10, 23). Cells were incubated with the corresponding virus, WNV strain NY99 or USUV strain SAAR-1776 (24), for 1 h at 37°C; viral inoculum was removed; and infected cells were incubated in culture medium containing 1% fetal bovine serum (time that was considered 1 h postinfection [p.i.]).

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Viral titer was determined 24 h p.i. by plaque assay in semisolid agarose medium using Vero cells (25). The multiplicity of infection (MOI) used in each experiment was expressed as PFU per cell and is indicated in the corresponding figure legend.

Drug treatments. 5-(Tetradecyloxy)-2-furoic acid (TOFA) and 3,3,14,14-tetramethylhexadecanedioic acid (MEDICA 16) were from Sigma (St. Louis, MO). Control cells were treated in parallel with the same amount of drug vehicle (dimethyl sulfoxide [DMSO]). Unless otherwise specified, drugs were added after the first hour of infection, when viral inoculum was replaced by medium containing 1% fetal bovine serum. Drug toxicity was examined by measuring the cellular ATP content with the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI).

Microscopy. Antibodies and procedures used for immunofluorescence and confocal microscopy, as well as sample processing for transmission electron microscopy, have been described previously (11). The analysis of fluorescence intensity was performed using ImageJ software (http://imagej.nih.gov/ij/).

Quantitative RT-PCR. Viral RNA was extracted from the supernatant of infected cultures with the Speedtools RNA virus extraction kit (Biotools, Madrid, Spain). For quantification of cell-associated viral RNA, supernatants from infected cells were removed, cell monolayers were subjected to three freeze-thaw cycles, and RNA was extracted as described above. The amount of viral RNA was determined by real-time fluorogenic reverse transcriptase PCR (RT-PCR) as reported previously (26). The forward primer 5′-CAGACCACGCTACGGCG-3′, the reverse primer 5′-CTAGGGCCGCGTGGG-3′, and the probe 5′-FAM (6-carboxyfluorescein)-TCTGCGGAGAGTGCAGTCTGCGAT-3′-BHQ-1 (black hole quencher 1) were used. Quantification was performed using the High Scriptools-Quantimix Easy Probes kit (Biotools) and Rotor-Gene RG-3000 equipment (Corbett Research). Genomic equivalents to PFU per milliliter were calculated by comparison with 10-fold serial dilutions of viral RNA extracted from previously titrated samples (27).

Lipid analysis. Vero cells were treated with 10 μg/ml TOFA or with DMSO and infected or not with WNV at a high MOI (50 PFU/cell) to ensure that all cells were initially infected. Cells were detached from the flasks and resuspended in phosphate-buffered saline (PBS) at 24 h p.i. (10). Infections were performed in serum-free medium to avoid possible biases introduced by lipids present in the serum. The number of cells in each sample was determined, and aliquots containing 10^6 cells were subjected to lipid extractions. Procedures for lipid extractions, identification, and quantification by mass spectrometry have been previously described (10, 28, 29). Annotation of lipid species followed the recommendations indicated in reference 30. Glycerophospholipids, diacylglycerol (DAG), triacylglycerol (TAG), and cholesteryl esters (CHOL) were annotated as "lipid subclass" followed by "total fatty acyl chain length:total number of unsaturated bonds" (e.g., PC 36:2). Plasmalogens were annotated as described above, except that "p" was added. Lysophospholipids were annotated as described above except that "lyso" was added. Sphingolipids were annotated as "lipid subclass" followed by "total fatty acyl chain length: total number of unsaturated bonds" (e.g., CER 24:1). If the sphingoid base residue was dihydrosphingosine, the lipid class contained a "dh" prefix. For ceramide derivatives containing sugar moieties, the lipid class contained a "hex" prefix to denote monohexosylceramides (glucosylceramides and galactosylceramides) or a "lac" prefix for lactosylceramides.

Data analysis. Data are presented as means ± standard deviations (SDs). Analysis of variance (ANOVA) and Student's *t* test were performed with SPSS 15 (SPSS Inc., Chicago, IL). Bonferroni's correction was applied for multiple comparisons. Statistically significant differences were considered at a P value of <0.05.

RESULTS

Effect of inhibition of ACC on cellular lipid content. TOFA is an inhibitor of ACC that has been used to evaluate the involvement of this enzyme in infections by a variety of viruses (18, 31–34). To determine its effect on the cellular lipid content, Vero cells were treated with TOFA for 24 h and the lipid content was analyzed by mass spectrometry and compared to that of control cells treated in parallel with the same amount of drug solvent (Fig. 1). These experiments were performed in serum-free medium to avoid possible interference of serum lipids in the analysis. Eighteen lipid classes (3 neutral lipids [NLs], 10 glycerophospholipids [GPLs], and 5 sphingolipids [SLs]) were included in the analysis (Fig. 1A). A significant decrease in the amount of NLs (triacyclglycerol [TAG], diacylglycerol [DAG], and cholesteryl esters [CHOL]) was observed in TOFA-treated cells (Fig. 1A). Among SLs, ceramide (CER), monohexosylceramide (hexCER), lactosylceramide (lacCER), sphingomyelin (SM), and dihydroSM (dhSM) were analyzed. The amount of CER and its derivatives containing sugar moieties (hexCER and lacCER) was significantly reduced in TOFA-treated cells. In contrast, the content of SM and dhSM was not significantly reduced by treatment with TOFA (Fig. 1A). To evaluate the effect of TOFA on the cellular GPLs, we analyzed the content of phosphatidylcholine (PC), 1-alkenyl-2-acyl-glycero-3-phosphocholine (referred to as plasmalogen-PC [p-PC; plasmenylcholine]), 1-acyl-glycero-3-phosphocholine (lyso-PC), lysoplasmenylcholine (lyso-p-PC), phosphatidylethanolamine (PE), 1-alkenyl-2-acyl-glycero-3-phosphoethanolamine (p-PE), 1-acyl-glycero-3-phosphoethanolamine (lyso-PE), lyso-p-PE, phosphatidylserine (PS), and 1-acyl-glycero-3-phosphoserine (lyso-PS). A significant decrease in the amount of PC, p-PC, PE, p-PE, and PS was noted in TOFA-treated cells, whereas no significant change in the lysolipids analyzed (lyso-PC, lyso-p-PC, lyso-PE, lyso-p-PE, and lyso-PS) was induced by treatment with TOFA. The lack of reduction in the amount of lysolipids can be explained because lysolipids are produced by the action of phospholipases (35) rather than by a biosynthetic process. Among the 18 lipid classes analyzed, a total of 208 different molecular species could be identified (Fig. 1B to H). From these species, 89 remained unaltered in TOFA-treated cells (42.8%) and a significant increase was observed in only three of the identified species (1.4%) that corresponded to lysolipids (Fig. 1H). Such an increase could likely correspond to a stress response including the activation of phospholipases (35). On the other hand, on 116 lipid species (55.8%) a significant reduction was noted, confirming that inhibition of ACC using TOFA has a major impact on lipid synthesis, reducing the content of a wide variety of cellular lipids.

TOFA inhibits infection by WNV and USUV. Vero cells were infected with WNV, and different concentrations of TOFA were added 1 h p.i. to avoid possible interference of the drug with virus entry. TOFA inhibited WNV multiplication in a dose-dependent manner (Fig. 2A and B). Both the production of infectious particles (Fig. 2A) and the release of genome-containing particles to the culture medium (Fig. 2B) were significantly reduced by TOFA. The toxicity of the drug was analyzed in parallel by determination of the cellular ATP content (Fig. 2C), confirming that the concentrations of TOFA tested inhibited WNV infection, exerting small effects on cell viability. A time course analysis of the production of infectious virus revealed that TOFA inhibited the multiplication of WNV also at time points as early as 12 h p.i. (Fig. 2D). Next, the effect of TOFA on WNV infection in other cell lines was tested. An inhibition similar to that observed on Vero cells (Fig. 2A and B) was noted in TOFA-treated HeLa cells (Fig. 2E and F). Considering that WNV is a neurotropic virus, the effect of TOFA was also assayed in the neuroblastoma cell line N2a. TOFA also inhibited

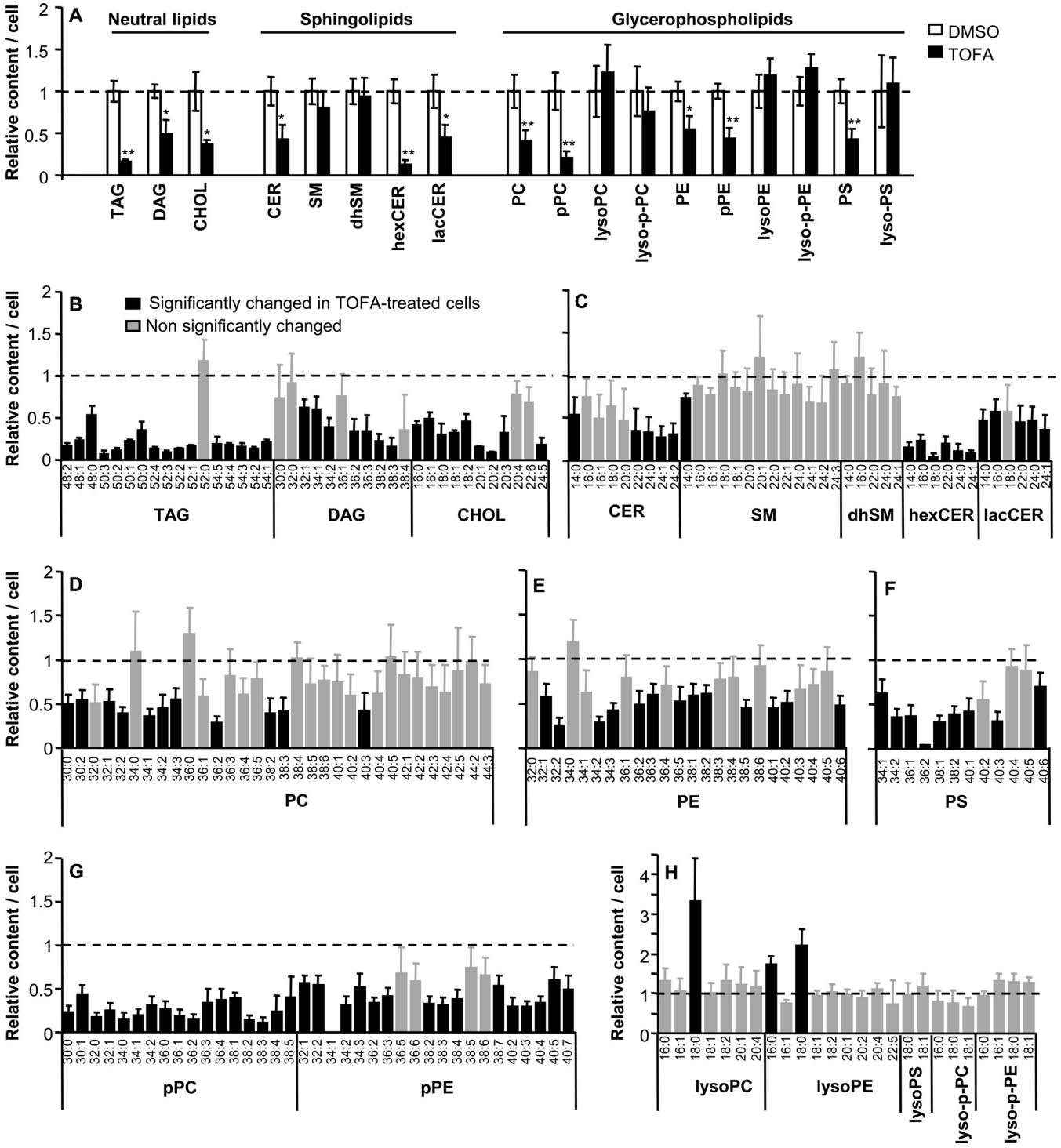

FIG 1 Alterations of cellular lipid content upon inhibition of ACC using TOFA. (A) Relative amounts of different classes of NLs, SLs, and GPLs in Vero cells treated with 10 μg/ml TOFA or not treated (DMSO) determined by mass spectrometry at 24 h after drug addition. The experiments were performed in serum-free medium. Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$. (B to H) Relative amounts of different lipid molecular species (NLs [B], SLs [C], PC [D], PE [E], PS [F], plasmalogens [G], and lysophospholipids [H]) in cells treated with TOFA as in panel A. Black bars denote molecular species significantly altered ($P < 0.05$) relative to control cells. Gray bars denote molecular species not significantly changed relative to untreated cells. Bars corresponding to untreated cells have been omitted from the figure for clarity. Dashed lines, mean value for each lipid in untreated cells. Data are presented as means ± SDs (three replicates).

the infection of WNV in N2a cells, showing also a minor impact on cell viability (Fig. 2G and H). When N2a cells were used to address the effect of TOFA addition to infected cultures at 1, 4, and 8 h p.i., a significant inhibition of WNV infection was observed in all cases (Fig. 2I), indicating that treatment with TOFA impaired WNV infection after virus entry. Even more, TOFA also significantly reduced the production of USUV, a related flavivirus, in Vero, HeLa, and N2a cells (Fig. 2J). These results suggest that the

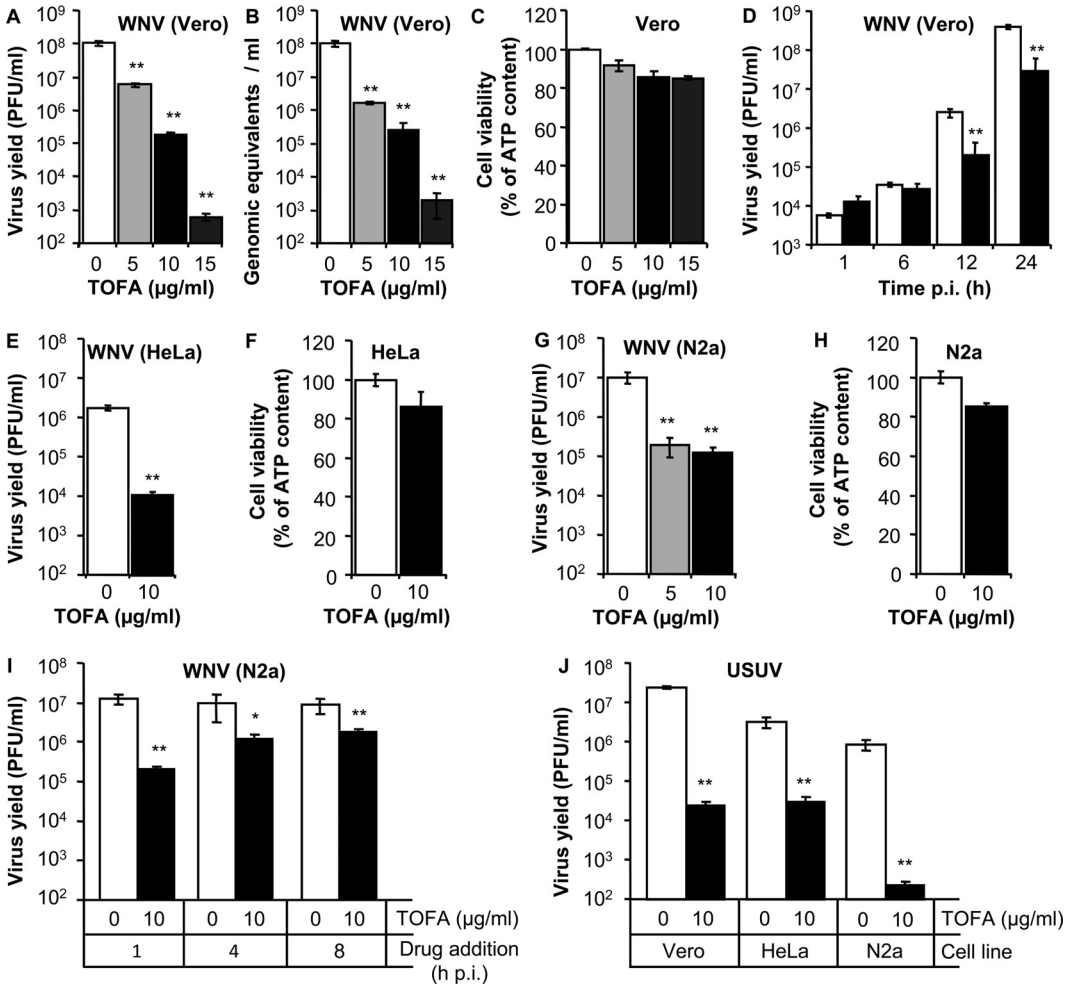

FIG 2 TOFA inhibits WNV and USUV multiplication. (A) Reduction of WNV infectious particle production in Vero cells treated with different concentrations of TOFA. Cells were infected with WNV (MOI of 0.5 PFU/cell), and virus yield in culture supernatant was determined by plaque assay at 24 h p.i. (B) Quantification by quantitative RT-PCR of genome-containing particles in culture supernatant of Vero cells infected as in panel A. (C) Evaluation of the toxicity of TOFA on Vero cells by determination of cellular ATP content 24 h posttreatment. (D) Time course analysis of virus production in TOFA-treated cells. Vero cells were infected with WNV (MOI of 1 PFU/cell) and treated with 10 μg/ml TOFA. The amount of infectious virus in the supernatant of infected cultures was determined by plaque assay at different times p.i. (E) Reduction of WNV infectious particle production (24 h p.i.) in HeLa cells infected (MOI of 0.5 PFU/cell) and treated with TOFA. (F) Evaluation of the toxicity of TOFA on HeLa cells 24 h posttreatment. (G) Reduction of WNV infectious particle production (24 h p.i.) in N2a cells infected (MOI of 0.5 PFU/cell) and treated with TOFA. (H) Evaluation of the toxicity of TOFA on N2a cells 24 h posttreatment. (I) Inhibition of WNV multiplication in N2a cells by adding TOFA at different times p.i. N2a cells were infected with WNV (MOI of 0.5 PFU/cell), and 10 μg/ml TOFA was added at the indicated time points. Virus yield was determined by plaque assay at 24 h p.i. (J) Inhibition of USUV infection in different cell lines using TOFA. Vero, HeLa, and N2a cells were infected with USUV (MOI of 0.5 PFU/cell) and treated with TOFA. Virus yield was determined by plaque assay at 24 h p.i. Data are presented as means ± SDs (three replicates). Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

inhibition of ACC can be also effective against other related flaviviruses.

TOFA inhibits the replication of WNV genome. The infection with WNV induces marked changes in the intracellular membrane architecture to create an appropriate microenvironment for viral replication (7, 11). Hence, we analyzed the effect of TOFA on Vero cells infected with WNV by means of transmission electron microscopy (Fig. 3A). Mock-infected cells treated with TOFA or not treated exhibited normal cellular architecture (Fig. 3A, upper panels). In contrast, control infected cells not treated with TOFA exhibited characteristic infection-associated ultrastructural alterations, such as convoluted membranes (CM), vesicle packets (VPs), and electron-dense virions (Vi) (Fig. 3A, lower left panel). In infected cells treated with TOFA, Vi could be recognized but properly arranged CM or VPs, such as those observed in control cells, could not be detected. Instead, disordered VP-like materials and small cumuli of membranous materials resembling CM were observed (Fig. 3A, lower right panel). Since the replication of the flavivirus genome takes place in the VP (4, 36), our observations could indicate that the alteration of cellular lipids induced by TOFA impaired the development of WNV replication complexes. To support this hypothesis, the amount of double-stranded RNA (dsRNA) intermediates (a well-characterized marker of flavivirus replication complex [4, 7]) was analyzed by immunofluorescence in infected cells treated or not treated with TOFA (Fig. 3B). As expected, no dsRNA was detected in uninfected cells, treated or not treated with TOFA (Fig. 3B, upper panels). In contrast, dsRNA was observed in both control and TOFA-treated cells in-

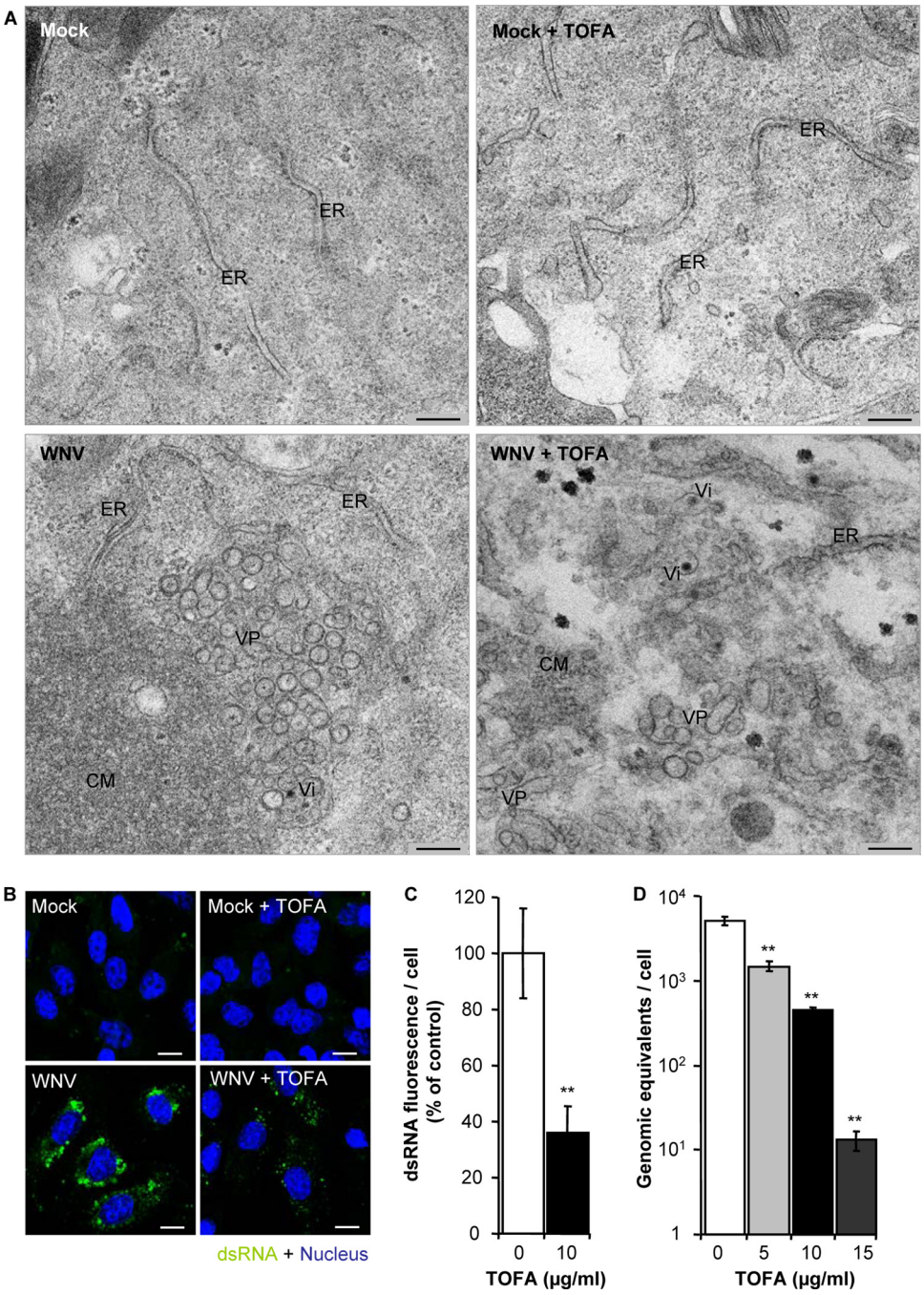

FIG 3 Effect of TOFA on replication of WNV. (A) Intracellular membrane rearrangements in WNV-infected Vero cells observed by transmission electron microscopy 24 h p.i. Upper panels show representative images of uninfected cells untreated (left) or treated with 10 μg/ml TOFA (right). Lower panels display representative images of infected cells (MOI, 10 PFU/cell) untreated (left) or treated with TOFA (right). VP, vesicle packet; Vi, virion; CM, convoluted membranes; ER, endoplasmic reticulum. Bars, 200 nm. (B) Visualization of intracellular dsRNA accumulation in cells infected with WNV (MOI, 10 PFU/cell) and treated with 10 μg/ml TOFA at 24 h p.i. Cells were fixed and processed for immunofluorescence using monoclonal antibody to dsRNA J2 and a secondary antibody coupled to Alexa Fluor 488 (green). Nuclei were stained with ToPro-3 (blue). Mock-infected cells processed in parallel are also included as a control. Bars, 10 μm. (C) Quantification of the fluorescence intensity of dsRNA in cells infected and treated with TOFA as shown in panel B. (D) Amount of cell-associated viral RNA in cell cultures infected with WNV (MOI of 0.5 PFU/cell) and treated with TOFA determined by quantitative RT-PCR at 24 h p.i. Data are presented as means ± SDs (three replicates). Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

fected with WNV. However, the intensity of the dsRNA fluorescence was lower in infected cells treated with TOFA than in control infected cells (Fig. 3B, lower panels). The quantification of this signal confirmed a statistically significant reduction in the cellular content of dsRNA intermediates in TOFA-treated cells (Fig. 3C). Furthermore, the analysis by quantitative RT-PCR revealed that treatment with TOFA induced a statistically significant dose-dependent reduction in the amount of cell-associated viral RNA

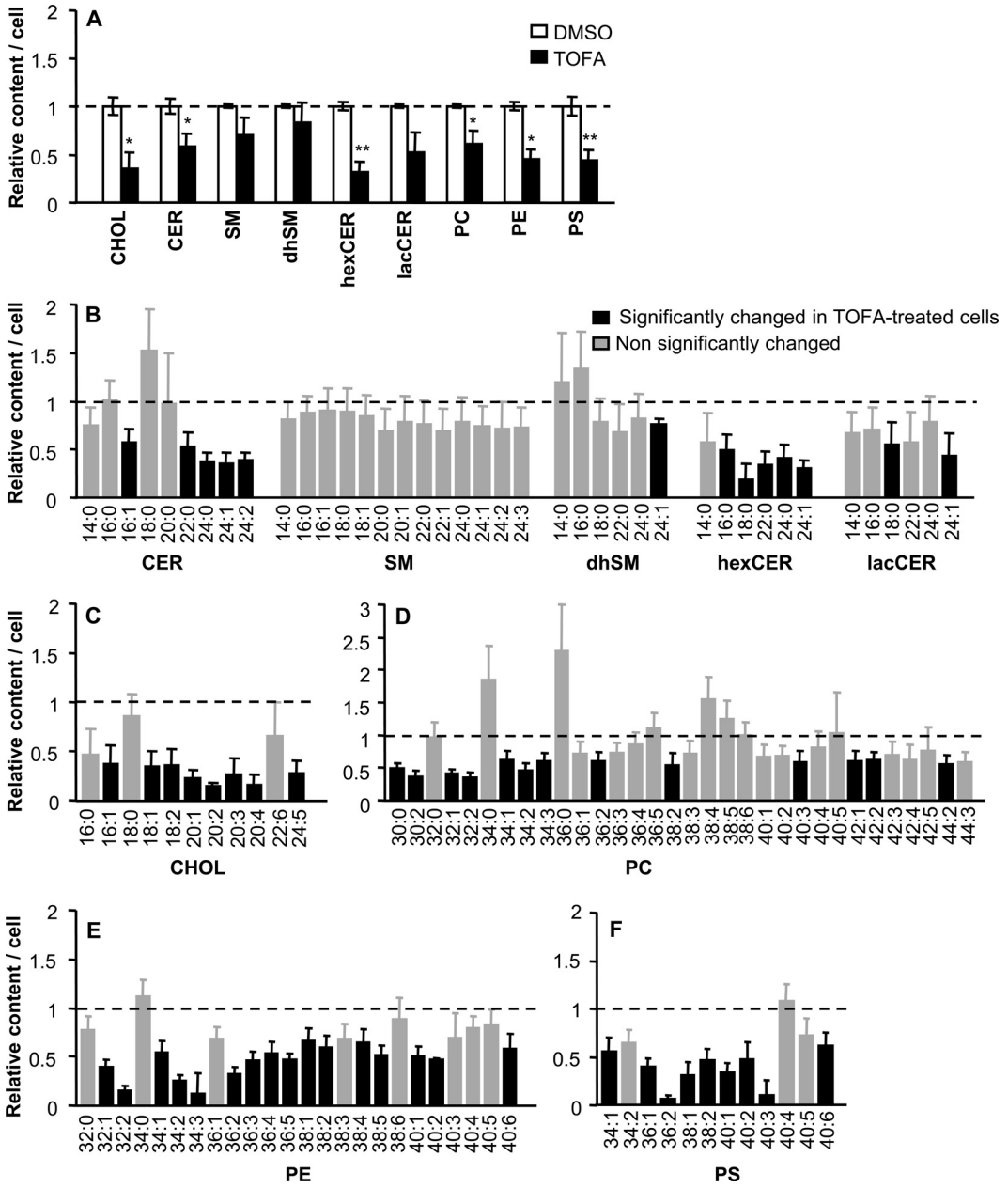

FIG 4 Reduction of cellular lipid content in WNV-infected cells treated with TOFA. (A) Relative amounts of different lipid classes in Vero cells infected with WNV (MOI of 50 PFU/cell) and treated with 10 μg/ml TOFA or not (DMSO), determined by mass spectrometry at 24 h p.i. The experiments were performed in serum-free medium. Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$. (B to F) Relative amounts of different lipid molecular species (SLs [B], CHOL [C], PC [D], PE [E], and PS [F]) in cells infected with WNV and treated with TOFA as in panel A. Black bars denote molecular species significantly altered ($P < 0.05$) relative to untreated infected cells. Gray bars denote molecular species not significantly changed relative to untreated infected cells. Bars corresponding to untreated infected cells have been omitted from the figure for clarity. Dashed lines, mean value for each lipid in untreated infected cells. Data are presented as means ± SDs (three replicates).

(Fig. 3D). Taken together, these results suggest that the alteration of cellular lipid content induced by TOFA impairs WNV replication complex assembly and hence viral replication.

TOFA reduces the lipid content of WNV-infected cells. The amount of lipids in WNV-infected cells was analyzed by mass spectrometry (Fig. 4). In this case, in order to simplify the analysis, we focused on 9 lipid classes, including representative members of NLs (CHOL), SLs (CER, SM, dhSM, hexCER, and lacCER), and GPLs (PC, PE, and PS). These lipid classes included important membrane components, some of which had been previously associated with different features of virus infection (8–10, 37, 38). Relative to infected cells not treated with the drug, a significant reduction in the content of CHOL, CER, hexCER, PC, PE, and PS was observed in infected cells treated with TOFA (Fig. 4A). Overall, these results supported the idea that, as observed in uninfected cells (Fig. 1), TOFA inhibited lipid synthesis in WNV-infected cells. Among the 9 lipid classes compared, a total of 119 different molecular species could be identified and analyzed (Fig. 4B to F).

A significant reduction of 59 molecular species was noted in infected cells treated with TOFA (49.6% of species analyzed), 48 of which (81% of species analyzed) were also significantly reduced in uninfected cells treated with TOFA (Fig. 1). These findings indicated that the lipid changes induced by TOFA were similar in both WNV-infected and uninfected cells.

MEDICA 16 inhibits the infection of WNV and USUV. Treatment of Vero cells with MEDICA 16, an ACC inhibitor different from TOFA (39), also resulted in a dose-dependent inhibition of the production of infectious WNV (Fig. 5A) at concentrations that did not severely impair the viability of the cells (Fig. 5B). The quantification of genome-containing particles by quantitative RT-PCR revealed that MEDICA 16 reduced the release of these viral particles to the culture medium (Fig. 5C). Remarkably, the extent of this inhibition was lower than the reduction produced in virus yield (compare Fig. 5A and C). This could indicate that treatment with MEDICA 16 was inducing the formation of noninfectious genome-containing WNV particles, a phenomenon that was not observed in infected cells treated with TOFA, in which the decrease in the production of infectious particles was similar to the reduction in the release of genome-containing particles (Fig. 2). Moreover, the concentration of 50 μg/ml MEDICA 16 that inhibited the production of infectious WNV and the release of genome-containing particles did not inhibit accumulation of cell-associated viral RNA within infected cells. However, a significant reduction of viral replication was observed at a higher concentration of 70 μg/ml (Fig. 5D). Nevertheless, MEDICA 16 strongly inhibited the infection with USUV (Fig. 5E), confirming that this hypolipidemic agent can also inhibit the multiplication of WNV-related flaviviruses.

DISCUSSION

Lipids play multifaceted roles during virus infection. In such scenarios, the pharmacological manipulation of cellular lipids can reduce virus infection by different mechanisms. These mechanisms include (i) the impairment of membrane rearrangements associated with viral replication, (ii) the generation of an unfavorable metabolic environment for virus replication, or (iii) the alteration of the viral envelope composition and the impairment of viral particle assembly (8, 17, 18, 31).

Treatment with TOFA reduced the synthesis of multiple cellular lipids, including those previously described to be involved in WNV infection, such as GPLs, SLs, and CHOL (8, 10, 15). Our electron microscopy results showed that TOFA induced a reduction in the amount of membrane rearrangements associated with WNV replication. In addition, quantitative RT-PCR analysis indicated that this drug reduced the synthesis of viral RNA, which was compatible with the reduction of the accumulation of dsRNA intermediates observed by immunofluorescence in infected cells treated with TOFA. Taken together, all these results suggest that the inhibition of lipid synthesis exerted by TOFA could impair the membrane rearrangements necessary for the replication of WNV. This mechanism of action would be similar to that recently reported for hepatitis C virus in cells treated with an inhibitor of the ACC (34). An additional point to be considered is that although treatment with TOFA reduced the amount of multiple lipid species, it also increased the amount of some lysolipids. Interestingly, the production of these lysolipids could be related to an activation of a stress response in TOFA-treated cells (35). The possible con-

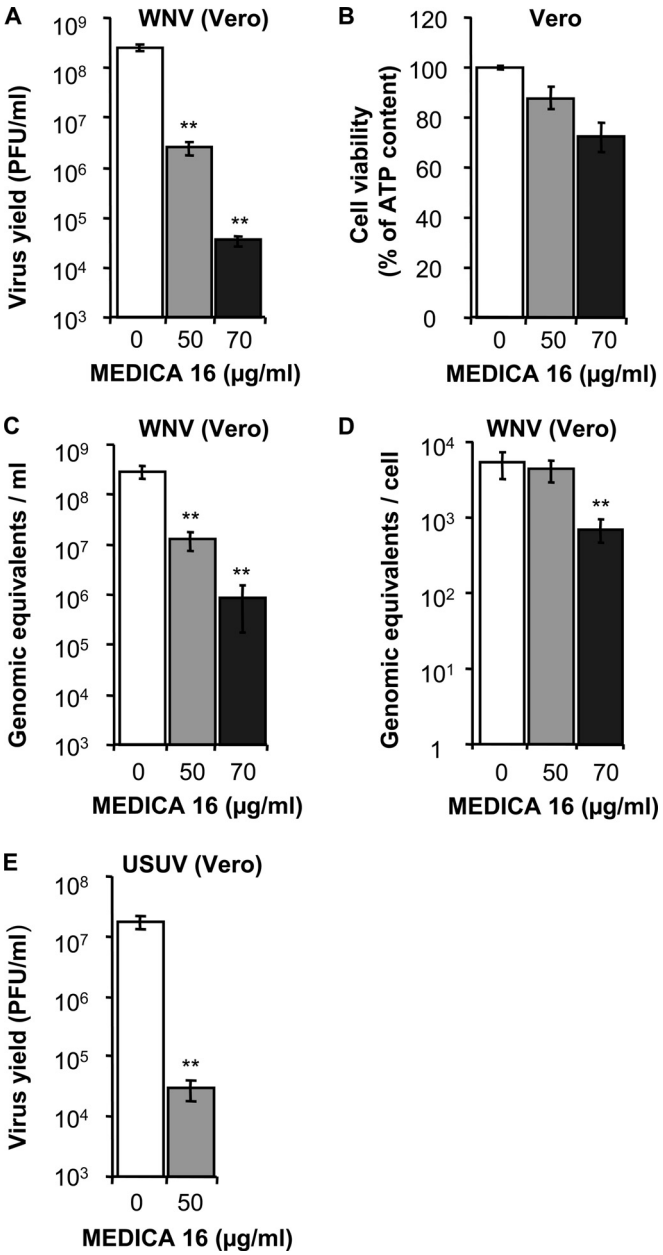

FIG 5 MEDICA 16 inhibits WNV and USUV multiplication. (A) Reduction of WNV infectious particle production in Vero cells treated with MEDICA 16. Cells were infected with WNV (MOI of 0.5 PFU/cell), and virus yield in culture supernatant was determined by plaque assay at 24 h p.i. (B) Evaluation of the toxicity of MEDICA 16 on Vero cells by determination of cellular ATP content 24 h posttreatment. (C) Determination by quantitative RT-PCR of the amount of genome-containing particles in culture supernatant of Vero cells infected with WNV (MOI of 0.5 PFU/cell) at 24 h p.i. (D) Amount of cell-associated viral RNA in cell cultures infected with WNV (MOI of 0.5 PFU/cell) and treated with MEDICA 16 determined by quantitative RT-PCR at 24 h p.i. (E) Inhibition of USUV infection in Vero cells using MEDICA 16. Vero cells were infected with USUV (MOI of 0.5 PFU/cell) and treated with MEDICA 16, and virus yield was determined by plaque assay at 24 h p.i. Data are presented as means ± SDs (three replicates). Statistically significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

tributions of this stress to the antiviral effect of TOFA still remain to be evaluated.

In the case of the other ACC inhibitor tested, MEDICA 16, the inhibition exerted by the lowest concentration of this drug assayed (50 μg/ml) seemed to rely mainly on a reduction of infectious virions rather than on RNA synthesis inhibition, although at a high concentration (70 μg/ml) this drug also inhibited RNA replication. Blockage of infectious virus production has been described for other viruses treated with drugs affecting lipid metabolism (31, 40). In this regard, it should be considered that replication and assembly of viral particles are coupled processes in flaviviruses (5). This may explain our results showing that the alteration of lipid metabolism could affect both processes. In contrast to what was described for other members of the *Flaviviridae* family (41), there is no evidence, to our knowledge, of palmitoylation of WNV proteins. Thus, palmitoylation does not seem to be an antiviral target of action for ACC inhibitors in WNV infection.

Although the strategy of manipulating a major cellular metabolic pathway such as lipid biosynthesis could induce multiple side effects *in vivo*, it has to be considered that current antivirals target other major biosynthetic pathways, such as nucleotide biosynthesis (for a discussion, see reference 18). Indeed, the clinical value of lipid-lowering agents such as the statins, which are cholesterol synthesis inhibitors broadly used against cardiovascular diseases, supports the feasibility of therapeutic interventions targeting the lipid metabolism (42). Consistently, hypolipidemic drugs targeting the ACC are being evaluated for the treatment of multiple human disorders from certain cancers to obesity and diabetes (20, 21) and even viral infections (18, 34, 43). In addition, the results obtained with hypolipidemic agents (i.e., nordihydroguaiaretic acid) that target cellular factors other than ACC also support the feasibility of lipid-based antiviral approaches (17, 44). In this context, the potential of ACC inhibitors reported here to impair the replication of different flaviviruses, such as WNV and USUV, could contribute to the development of broad-spectrum antiflaviviral drugs.

In this work, we have provided further evidence supporting the idea that pharmacological manipulation of the lipid biosynthetic route can impair flavivirus infection. Specifically, we have identified the ACC as a druggable target to be considered for antiviral development against WNV and related flaviviruses.

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Artículo III

Merino-Ramos T, Jiménez de Oya A, Saiz JC, Martín-Acebes MA. "Antiviral activity of Nordihydroguaiaretic acid and its derivative tetra-O-methyl nordihydroguaiaretic acid against West Nile virus and Zika virus"**.** Antimicrob Agents Chemother. 2017 May 15 pii: AAC.00376-17. doi: 10.1128/AAC.00376-17.

Siguiendo nuestras previas investigaciones, el Artículo III se centra en el análisis de la vía de las proteínas de unión a elementos reguladores del esterol (SREBP), ya que éstas son los principales factores de transcripción que regulan la biosíntesis y la homeostasis lipídica en mamíferos. Éste estudio revela la implicación de la vía SREBP sobre la infección por VNO. De éste modo, una droga hipolipidémica (el ácido nordihidroguaiarético o NDGA) y su derivado metilado (el tetra-*O*-methil nordihidroguaiarético o M_4N) inhiben la multiplicación del VNO (linajes 1 y 2), así como del virus Zika. Es más, otros inhibidores de la vía SREBP estructuralmente no relacionados, como son el PF-429242 y la Fatostatina, también reducen la multiplicación del VNO, apoyando que la vía SREBP podría constituir una posible diana terapéutica para el desarrollo de antivirales contra la infección por flavivirus. Además, el tratamiento con PF-429242, y Fatostatina, al igual que ocurría con NDGA y M_4N, también inhibían la multiplicación del flavivirus Zika.

En el transcurso de estas investigaciones llevé a cabo la mayoría del trabajo experimental y contribuí al diseño de una parte significativa de los experimentos. Realicé las infecciones de células (Vero) con virus (VNO de linaje 1 y 2) y su tratamiento con concentraciones crecientes de distintos fármacos (NDGA, M4N, Resveratrol, PF-429242, y Fatostatina), determinando la toxicidad de los mismos en las distintas células utilizadas y titulando las progenies víricas obtenidas. Obtuve y cuantifiqué el ARN de doble cadena mediante inmunofluorescencia en células infectadas, tratadas o no con fármaco. Llevé a cabo análisis, mediante *Western blot*, de la expresión de la glicoproteína E del VNO en células tratadas o no con fármacos. Así mismo, purifiqué el ARN viral y lo analicé por RT-PCR cuantitativa. Finalmente llevé a cabo parte del análisis de los datos y contribuí a la escritura del artículo detallando los resultados obtenidos.

Article III

Merino-Ramos T, Jiménez de Oya A, Saiz JC, Martín-Acebes MA. "Antiviral activity of Nordihydroguaiaretic acid and its derivative tetra-O-methyl nordihydroguaiaretic acid against West Nile virus and Zika virus"**.** Antimicrob Agents Chemother. 2017 May 15 pii: AAC.00376-17. doi: 10.1128/AAC.00376-17.

Following our previous approaches, **Article III** focus on the sterol regulatory element binding proteins (SREBPs) pathway, as they are the main transcription factors that regulate lipid biosynthesis and lipid homeostasis in mammals. This study, unveils the involvement of the SREBP pathway on WNV infection. Thus, a hypolipodemic drug (nordihydroguaiaretic acid, NDGA) and its methylated derivative (tetra-*O*-methyl nordihydroguaiaretic, M_4N) inhibited WNV (lineage 1 and 2) and ZIKV multiplication. Even more, other structurally unrelated inhibitors of the SREBP pathway, such as PF-429242 and Fatostatin, also reduced WNV multiplication, supporting that the SREBP pathway could constitute a druggable target suitable for antiviral intervention against flavivirus infection. Moreover, treatment with PF-429242, and Fatostatin, as occurred with NDGA and M_4N, also inhibited the multiplication of the mosquito-borne flavivirus Zika virus (ZIKV).

In the course of the research, I developed most of the experimental work and I contributed to the experimental design. I made the cells infection (Vero) with both WNV lineage 1 and 2 strains, and its treatment with increasing concentrations of different drugs (NDGA, M4N, Resveratrol, PF-429242, and Fatostatin). Previously I determined drug toxicity in the same cell line and afterwards, I made the viral titration. I obtained and quantified dsRNA intermediates by immunofluorescence in infected cells treated or not with drugs. I carry out *Western blot* analysis of E glycoprotein expression in cells treated or not with drugs. Likewise, I made viral RNA extractions and analyzed them by quantitative RT-PCR. Finally, I did a part of the data analysis and contributed to the article writing, detailing the obtained results.

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ANTIVIRAL AGENTS

Antiviral Activity of Nordihydroguaiaretic Acid and Its Derivative Tetra-*O*-Methyl Nordihydroguaiaretic Acid against West Nile Virus and Zika Virus

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ABSTRACT Flaviviruses are positive-strand RNA viruses distributed all over the world that infect millions of people every year and for which no specific antiviral agents have been approved. These viruses include the mosquito-borne West Nile virus (WNV), which is responsible for outbreaks of meningitis and encephalitis. Considering that nordihydroguaiaretic acid (NDGA) has been previously shown to inhibit the multiplication of the related dengue virus and hepatitis C virus, we have evaluated the effect of NDGA, and its methylated derivative tetra-*O*-methyl nordihydroguaiaretic acid (M_4N), on the infection of WNV. Both compounds inhibited the infection of WNV, likely by impairing viral replication. Since flavivirus multiplication is highly dependent on host cell lipid metabolism, the antiviral effect of NDGA has been previously related to its ability to disturb the lipid metabolism, probably by interfering with the sterol regulatory element-binding proteins (SREBP) pathway. Remarkably, we observed that other structurally unrelated inhibitors of the SREBP pathway, such as PF-429242 and fatostatin, also reduced WNV multiplication, supporting that the SREBP pathway may constitute a druggable target suitable for antiviral intervention against flavivirus infection. Moreover, treatment with NDGA, M_4N, PF-429242, and fatostatin also inhibited the multiplication of the mosquito-borne flavivirus Zika virus (ZIKV), which has been recently associated with birth defects (microcephaly) and neurological disorders. Our results point to SREBP inhibitors, such as NDGA and M_4N, as potential candidates for further antiviral development against medically relevant flaviviruses.

KEYWORDS West Nile virus, antiviral agents, flavivirus, lipid, ZIKV

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T.M.-R. and N.J.D.O. contributed equally to this article.

West Nile virus (WNV) is a member of the *Flavivirus* genus within the *Flaviviridae* family. Its genome is composed by a single-stranded RNA molecule of positive polarity about 11,000 nucleotides in length (1). This mosquito-borne flavivirus is distributed worldwide and causes outbreaks of febrile illness and meningoencephalitis (2). Although WNV poses a health threat, there is still neither a vaccine nor an antiviral therapy licensed for human use. Apart from WNV, the *Flavivirus* genus includes other well-known pathogens, such as yellow fever virus, dengue virus (DENV), Japanese encephalitis virus, tick-borne encephalitis virus, and Zika virus (ZIKV). ZIKV has recently gained attention because it is responsible for a global outbreak infecting millions of people and has been associated with birth defects (microcephaly) and neurological disorders like Guillain-Barré syndrome (3, 4). Similar to WNV, there are neither specific AQ: A drugs nor vaccines licensed against ZIKV.

Recent advances have revealed that positive-stranded RNA viruses, including the flaviviruses, rearrange host cell lipid metabolism and coopt for cellular lipids to com-

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plete their life cycle (5–7). Considering this dependence on lipid metabolism during flavivirus infection, pharmacological modification of the lipid metabolic pathways appears to be a proper strategy to impair flaviviral replication (7, 8). Along these lines, the hypolipidemic drug nordihydroguaiaretic acid (NDGA) has been reported to inhibit replication of the flavivirus DENV (9). In addition, NDGA also inhibited the replication of hepatitis C virus (HCV), a member of the *Hepacivirus* genus within the *Flaviviridae* family (10), thus becoming an interesting candidate for broad antiviral development against flaviviruses and related viruses. NDGA is a phenolic compound and the main metabolite of the desert shrub *Larrea tridentata*. Nowadays, NDGA is being evaluated to treat a wide variety of illnesses, including diabetes, pain, inflammation, infertility, rheumatism, arthritis, and gallbladder and kidney stones (11, 12). Remarkably, a synthetic methylated derivative of NDGA, termed tetra-*O*-methyl nordihydroguaiaretic acid (M_4N, Terameprocol, or EM-1421), which retains a high similarity in the molecular structure with its precursor, is currently in phase I/II clinical trials in patients with advanced cancer (13–15). However, to our knowledge, the potential antiviral effect of M_4N has not been assessed against any flavivirus.

In this work, we have evaluated the antiviral effect of NDGA and its M_4N derivative on the infection of medically relevant flaviviruses. Our results showed that both compounds inhibited WNV and ZIKV infection.

RESULTS

NDGA and M_4N inhibit WNV infection. The antiviral activity against WNV of NDGA and its synthetic methylated derivative M_4N was explored (Fig. 1A). Vero cells were infected and treated with different concentrations of the drugs (10 or 35 μM) at 1 hour postinfection (h p.i.), and the virus yield was determined by plaque assay at 24 h p.i. Both NDGA and M_4N reduced significantly the virus yield in a dose-dependent manner when cells were infected with a highly neurovirulent WNV strain from genetic lineage 1 (WNV NY99) representative of the virus that is currently circulating in the American continent (Fig. 1B and C). In a similar manner, both NDGA and M_4N also reduced significantly the virus yield of a WNV strain from genetic lineage 2 (WNV SRB-Novi Sad/12) representative of the highly neurovirulent virus that has recently colonized the European continent (Fig. 1D and E). The lack of toxicity of the drug concentrations utilized was analyzed in parallel by determination of the cellular ATP content (Fig. 1F and G). Results showed no statistically significant reduction of the cellular ATP content at the drug concentrations used, confirming that the reduction of viral yield was not associated with major cytotoxic effects of the drugs. Considering that there were no marked differences on the antiviral effect of NDGA and M_4N between the two WNV isolates analyzed (Fig. 1B to E), the WNV NY99 strain was selected for subsequent experiments.

Inhibition of WNV infection by M_4N is not related to a virucidal effect. To evaluate a possible virucidal effect of NDGA and M_4N, WNV ($\sim 1.5 \times 10^9$ PFU) was preincubated with the compounds for 1 h at 37°C in culture medium and then titrated to determine the remaining infectivity. A significant reduction (Fig. 2A) was observed only when NDGA was tested at the highest concentration (35 μM); however, this was lower than that observed in the virus yield assays (Fig. 1A), thus suggesting that the inactivation of the virions by NDGA was not primarily related to a virucidal effect. No significant reduction of WNV infectivity was noticed in this assay when M_4N was tested (Fig. 2B), indicating that this compound does not exhibit a virucidal effect against WNV.

NDGA and M_4N inhibit genome replication of WNV. To identify the step that was mainly affected by NDGA and M_4N, WNV infection was analyzed by quantitative reverse transcriptase PCR (RT-PCR). Both drugs significantly inhibited the release of WNV genome-containing particles to the culture medium (Fig. 3A and B). Moreover, NDGA and M_4N significantly reduced the amount of cell-associated viral RNA, especially at 35 μM (Fig. 3C and D). Overall, these observations support that the reduction in the release of genome-containing particles was produced by a decrease in viral replication. The amount of double-stranded RNA (dsRNA) intermediates, which provide a good indica-

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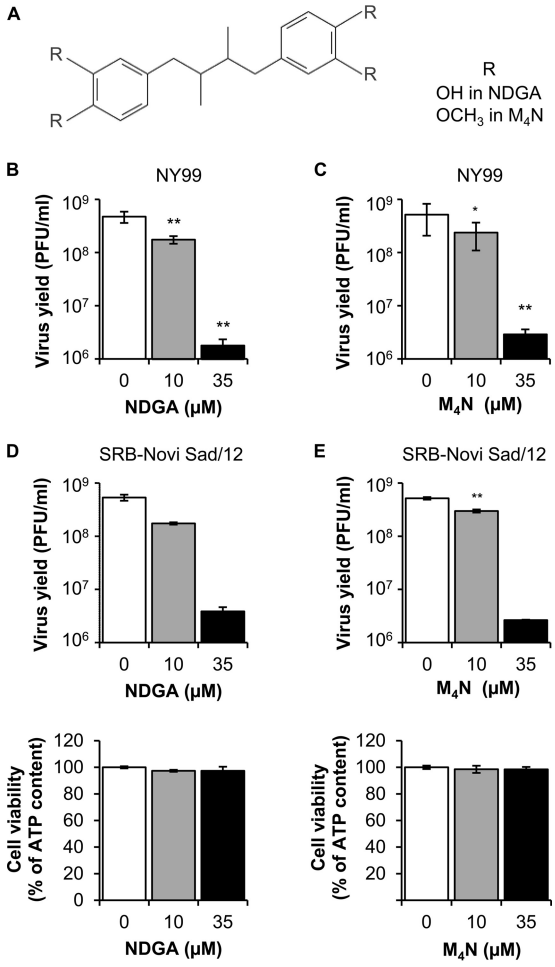

FIG 1 NDGA and its synthetic derivative M_4N inhibit WNV infection. (A) Chemical structure of NDGA and M_4N. R corresponds to OH in NDGA or OCH_3 in M_4N. (B and C) Reduction of WNV infection in Vero cells treated with NDGA (B) or M_4N (C). Cells were infected with WNV lineage 1 strain NY99 (MOI of 1 PFU/cell), and virus yield in culture supernatant was determined by plaque assay at 24 h p.i. (D and E) Inhibition of WNV lineage 2 infection in Vero cells treated with NDGA (D) or M_4N (E). Cells were infected with WNV SRB-Novi Sad/12 (MOI of 1 PFU/cell), and virus yield in culture supernatant was determined by plaque assay at 24 h p.i. (F and G) Evaluation of the cytotoxicity of NDGA (F) and M_4N (G) on Vero cells by determination of ATP content 24 h posttreatment. Data are presented as means ± SDs (n = 3 to 6). Statistically significant differences are indicated. *, $P < 0.05$; **, $P < 0.005$.

tor of flavivirus replication (16, 17), was also analyzed by immunofluorescence (Fig. 3E). Concordant with previous results, a major decrease in the amount of dsRNA was observed in cells treated with NDGA or M_4N in comparison to that in untreated cells. The quantification of this signal (Fig. 3F) confirmed this observation, supporting that both NDGA and M_4N inhibited WNV genome replication. Western blot analysis also revealed a reduction in the level of the viral E protein within infected cells treated with 35 μM NDGA (34% of control) or M_4N (43% of control) (Fig. 3G).

The effect of NDGA and M_4N on viral morphogenesis and particle egression was evaluated using a cell line that expresses WNV structural glycoproteins and constitutively secretes virus-like particles (termed recombinant subviral particles [RSPs]) without the need for viral replication (18, 19). For this experiment, cells were incubated for 4 h with the compounds, a time that has been previously shown to be sufficient for the identification of compounds that impair WNV biogenesis (19), and the amount of RSPs in the supernatant was analyzed. Immunoblot analysis of the supernatant using anti-E antibody revealed that no inhibition of RSP egress was observed when cells were treated with either NDGA or M_4N (Fig. 3H). However, the inhibitor of secretion brefeldin

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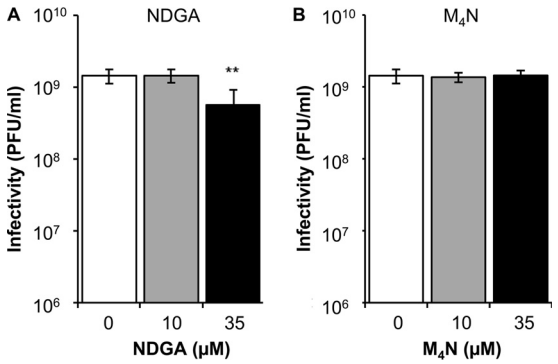

FIG 2 Evaluation of the direct effect of NDGA and M_4N on the infectivity of WNV. WNV NY99 (~1.5×10^9 PFU) was treated with NDGA (A) or M_4N (B) for 1 h at 37°C in culture medium. Then, the infectivity in each sample was determined by plaque assay. Data are presented as means ± SDs ($n = 4$). Statistically significant differences are indicated. **, $P < 0.005$.

A (BFA), included as a control, significantly inhibited the release of RSPs into the culture medium. Taken together, these results support that both NDGA and its derivative M_4N impair WNV infection by reducing genome replication rather than virion morphogenesis or particle egress.

Resveratrol, PF-429242, and fatostatin reduce WNV infection. The antiviral effect of NDGA against DENV and HCV has been associated with the inhibition of lipogenesis (9, 10). Specifically, in the case of HCV, the antiviral effect of NDGA was related to its ability to impair the sterol regulatory element-binding proteins (SREBPs) pathway (10). The SREBPs are the main transcription factors that regulate lipid biosynthesis and lipid homeostasis in mammals (20, 21). To evaluate the involvement of the SREBP pathway on WNV infection, we selected resveratrol, PF-429242, and fatostatin (Fig. 4A), which are structurally unrelated compounds that inhibit different components or regulators of the SREBP pathway (22–25). Treatment with resveratrol resulted in a dose-dependent inhibition of WNV infectious particle production (Fig. 4B). Likewise PF-429242 (Fig. 4C) and fatostatin (Fig. 4D) showed statistically significant suppression of infectious viral titers in a dose-dependent manner. In addition to its effect on the SREBP pathway, NDGA can also inhibit 5-lipoxygenase (5-LOX) activity *in vitro* (26, 27). Therefore, to discard that the antiviral effect of NDGA and M_4N was due to 5-LOX inhibition, the inhibitor of 5-LOX zileuton was used (28). No inhibition of virus yield was observed when cells were treated with zileuton (Fig. 4E). These results suggested that whereas the infection of WNV was sensitive to the inhibition of the SREBP pathway, it was independent of 5-LOX activity. The lack of toxicity of all of the drug concentrations utilized was analyzed in parallel by determination of ATP content (Fig. 4F to I). All of the drug concentrations tested displayed cell viabilities higher than 80% of that of control cells, further supporting that the reductions of viral yields exerted by resveratrol, PF-429242, and fatostatin were not associated with cytotoxic effects of the drugs.

NDGA, M_4N, PF-429242, and fatostatin inhibit ZIKV infection. The current explosive spread of ZIKV propelled us to test whether the drugs used against WNV could be used as well to decrease ZIKV infection. To achieve this goal, the effects of NDGA, M_4N, and the two hypolipidemic drugs, PF-429242 and fatostatin, on the multiplication of ZIKV PA259459, isolated from a human patient in Panama in 2015, were examined. As described for WNV, a statistically significant reduction in viral yield was observed for ZIKV when cells were treated with NDGA, M_4N, PF-429242, or fatostatin (Fig. 5). These results confirmed the antiviral effects of these compounds, including M_4N, against other medically relevant flaviviruses such as ZIKV.

Selectivity indexes of NDGA and M_4N against WNV and ZIKV. The evaluation of the balance between safety and efficacy of drugs is important for drug discovery and development (29), so the selectivity indexes (SIs) of NDGA and M_4N were calculated for WNV and ZIKV (Table 1). This index determines the relative effectiveness of the drug in

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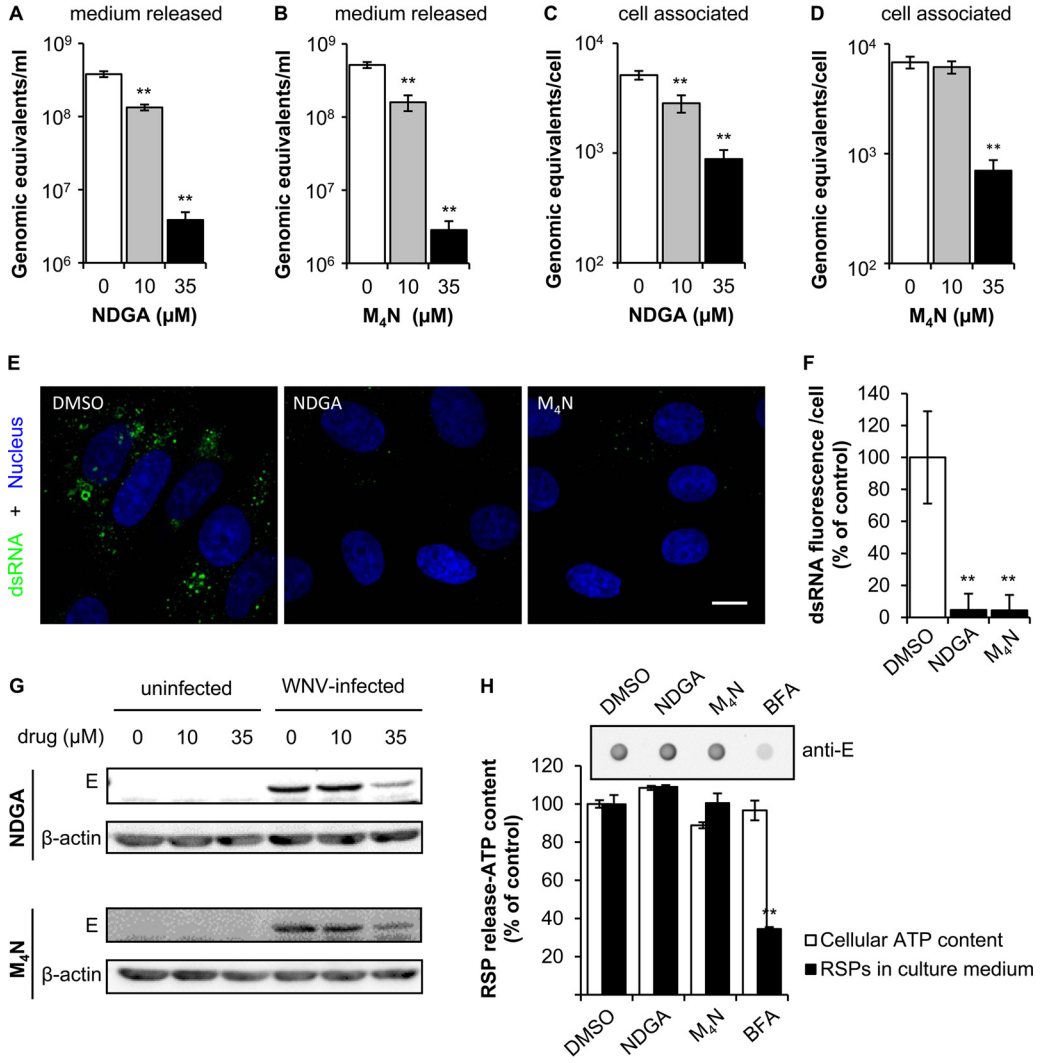

FIG 3 NDGA and M_4N inhibit WNV replication. (A and B) Reduction of genome-containing particles in culture supernatant of Vero cells treated with NDGA (A) or M_4N (B). Cells were infected with WNV NY99 (MOI of 1 PFU/cell), and the amount of genome-containing particles in culture supernatant was determined by quantitative RT-PCR at 24 h p.i. (C and D) Amount of cell-associated viral RNA in cell cultures infected with WNV and treated with NDGA (C) or M_4N (D) as described in A and B. The amount of cell-associated RNA in each sample was normalized by quantification of rRNA 18S. (E) Visualization of intracellular dsRNA accumulation in cells infected with WNV NY99 (MOI of 10 PFU/cell) and treated with 35 μM NDGA or 35 μM M_4N. Infected cells treated with drug vehicle (DMSO) were included as a control. Cells were fixed and processed for immunofluorescence using J2 monoclonal antibody to dsRNA and a secondary antibody coupled to Alexa Fluor 488 (green). Nuclei were stained with To-Pro-3 (blue). Bar, 10 μm. (F) Quantification of the fluorescence intensity of dsRNA in cells infected with WNV and treated with 35 μM NDGA or M_4N as shown in E (n = 89, 36, and 39 cells analyzed for DMSO, DDGA, and M_4N, respectively). (G) Western blot analysis of E glycoprotein expression in cells treated with NDGA or M_4N. Vero cells were infected, or not, with WNV NY99 (MOI of 1 PFU/cell) and treated with different concentrations of NDGA or M_4N. Membrane was retested against a β-actin antibody as a control for protein loading. (H) RSP release into the culture medium by HeLa3-WNV cells treated (4 h) with 35 μM NDGA, 35 μM M_4N, or 5 μg/ml BFA was analyzed by enzyme-linked immunodot assay using a monoclonal antibody against E glycoprotein. The graph displays the quantification of the amount of RSPs released. For each drug, the cellular ATP content is also depicted as an indicator of the cell viability upon drug treatment. Data are presented as means ± SDs (n = 3 to 6). Statistically significant differences are indicated. **, $P < 0.005$.

inhibiting viral replication compared to inducing cytotoxicity. SI was determined by quantifying the relation between the 50% cytotoxic concentration (CC_{50}; calculated as the concentration that resulted in the reduction of 50% of the amount of cellular ATP) and the 50% inhibitory concentration (IC_{50}; calculated as the concentration of drug at which viral infection was inhibited by 50%). Both compounds showed IC_{50}s in the one digit micromolar range for WNV and ZIKV. Since the CC_{50} of M_4N was about 1 order of magnitude higher than that of NDGA (Table 1), the SI of M_4N was improved (up to a value of >100) compared to that of NDGA for both WNV and ZIKV.

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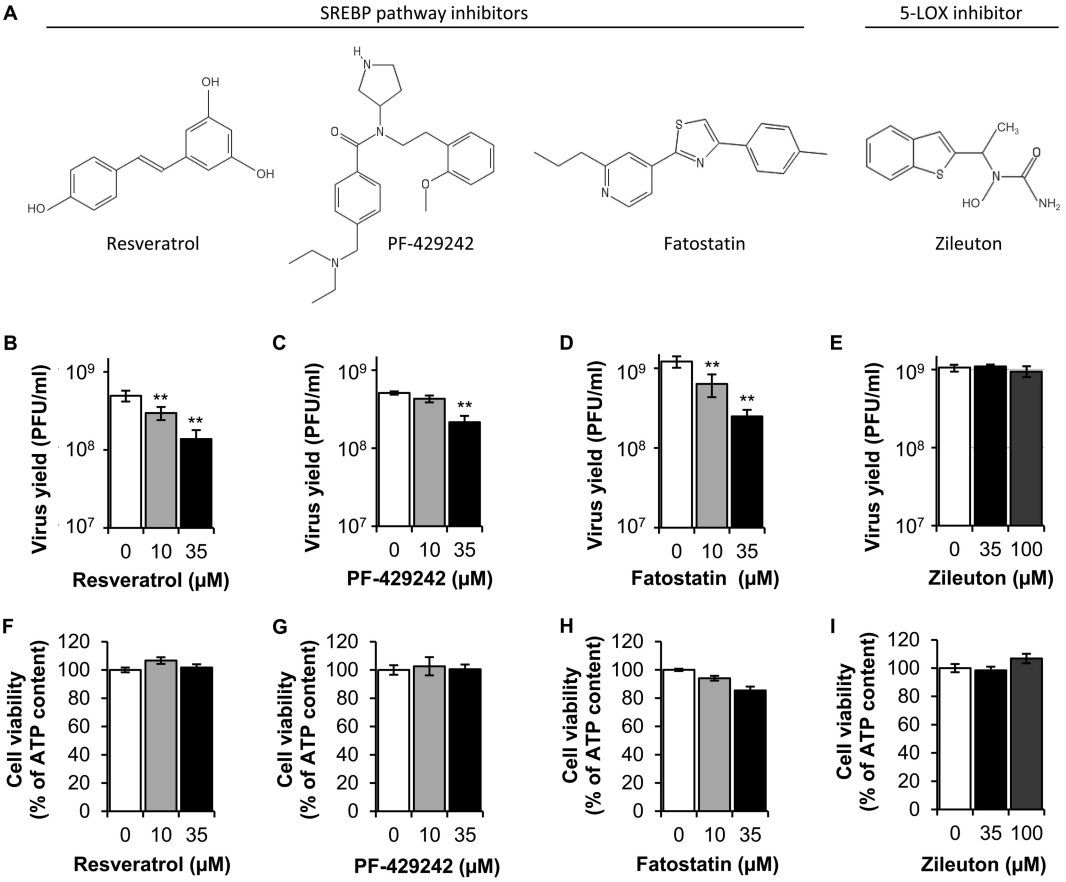

FIG 4 Different inhibitors of the SREBP pathway reduce WNV infection. (A) Chemical structure of the SREBP pathway inhibitors tested: resveratrol, PF-429242, and fatostatin. The structure of zileuton, a 5-LOX inhibitor, is also depicted. (B to E) Quantification of WNV infectious particle production in Vero cells infected with WNV NY99 (MOI of 1 PFU/cell) and treated with resveratrol (B), PF-429242 (C), fatostatin (D), or zileuton (E). Virus yield in culture supernatant was determined by plaque assay at 24 h p.i. (F to I) Evaluation of the cytotoxicity of resveratrol (F), PF-429242 (G), fatostatin (H), and zileuton (I) on Vero cells by determination of ATP content 24 h posttreatment. Data are presented as means ± SDs ($n = 4$). Statistically significant differences are indicated. **, $P < 0.005$.

DISCUSSION

Due to their inability to perform their own lipid synthesis, flaviviruses are forced to coopt for cellular lipids to complete their replication cycles. Accordingly, pharmacological manipulation of cellular lipid metabolism is rising as an alternative potential strategy to combat these pathogens (7, 8). Along these lines, we have shown that the hypolipidemic compound NDGA and its synthetic derivative M_4N exhibit potent antiviral activity against WNV and ZIKV. This antiviral effect of NDGA is consistent with results obtained for DENV and HCV (9, 10). It has to be remarked that the antiviral effects of NDGA or M_4N also have been observed against viruses from other viral families, such as herpes simplex virus, human immunodeficiency virus, human papillomavirus, cowpox virus, and vaccinia virus (30–34). Regarding the mechanism behind the antiviral activity of NDGA, in the case of HCV, it was related to an inhibition of the SREBP pathway (10). Consistent with this hypothesis, our results showed that a panel of structurally unrelated inhibitors of the SREBP pathway, such as resveratrol, fatostatin, and PF-429242, also reduced the multiplication of WNV and ZIKV. These results also supported previous studies that point to the importance of the SREBP pathway for infections of flavivirus and hepacivirus (9, 10, 35). The reduction observed for WNV using the SREBP inhibitors resveratrol, fatostatin, and PF-429242 was lower than that observed for NDGA and M_4N (compare Fig. 1 and 3), which may suggest that the inhibition of the SREBP pathway is not the only mechanism of action of NDGA and M_4N on WNV replication. In the case of the flavivirus DENV, it was proposed that NDGA

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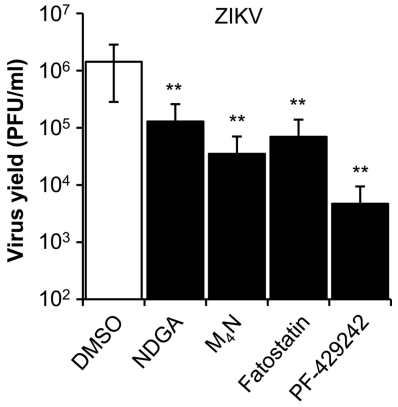

FIG 5 Reduction of ZIKV infectious particle production in Vero cells treated with NDGA, M_4N, fatostatin, or PF-429242. Cells were infected with ZIKV PA259459 (MOI of 1 PFU/cell), treated with 35 μM of each drug, or not (DMSO), and the virus yield in culture supernatant was determined by plaque assay at 24 h p.i. Data are presented as means ± SDs ($n = 3$). Statistically significant differences between control (DMSO) and drug-treated cells are indicated. **, $P < 0.005$.

inhibited viral infection by the following two different means: through viral genome replication reduction and virion assembly inhibition (9). In the present report, quantitative RT-PCR data and immunofluorescence analyses supported that both NDGA and M_4N also impaired the replication of the WNV genome. On the contrary, when the effects of NDGA and M_4N were directly evaluated on WNV morphogenesis and egress by measuring the production of virus-like particles, no significant reduction was noticed. Therefore, these results support that, in the case of WNV, the inhibitory effects of NDGA and M_4N were more likely due to an inhibition of viral genome replication rather than to an impairment of virion morphogenesis. Although we cannot exclude that the drugs also may be acting on entry or viral RNA translation, considering that the entry of flaviviruses into host cells is a very fast process that includes viral fusion within 5 to 10 min p.i. (36, 37) and that in our experimental approach the drugs were added 1 h p.i., it is reasonable to think that their effect is not primarily due to an inhibition of early infection steps. In addition, as no virucidal effect of M_4N, and only a slight one of NDGA, was noticed at the highest concentration tested (being markedly lower than the inhibition caused in the viral yield), it is also unlikely that this will be the main mechanism of inhibition of these compounds. The difference in the virucidal ability observed between the two compounds may be because M_4N is a methylated derivative of NDGA. In fact, supporting this view, the direct effect of other phenolic compounds on viral particles of the related hepatitis C virus has been linked to the presence of certain hydroxyl groups within the molecules tested (38).

In our experiments, NDGA and M_4N showed similar antiviral activities against WNV and ZIKV at concentrations near to those that can be detected in tissues and plasma of treated animals (39, 40). However, our CC_{50} values for each compound evidenced that M_4N displayed a reduced toxicity in comparison to that of NDGA. In fact, despite the high similarity in the molecular structures of NDGA and M_4N, it has been described that the median lethal dose of M_4N for mice was greater than 1,000 mg/kg while that of

TABLE 1 Selectivity indexes of NDGA and M_4N against WNV NY99 and ZIKV PA259459

		WNV NY99		ZIKV PA259459	
Compound	CC_{50}[a] (μM)	IC_{50}[b] (μM)	SI (CC_{50}/IC_{50})	IC_{50} (μM)	SI (CC_{50}/IC_{50})
NDGA	162.1	7.9	20.5	9.1	17.8
M_4N	1,071.0	9.3	115.2	5.7	187.9

[a]CC_{50} is the concentration that results in the reduction of 50% of the amount of cellular ATP after 24 h of treatment.

[b]IC_{50} is the concentration of drug at which virus yield (MOI of 1 PFU/cell) is inhibited by 50%. Virus yield was determined by plaque assay 24 h p.i.

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NDGA was only 75 mg/kg (12, 14, 39). Moreover, it has been reported that patients can tolerate higher doses of M_4N with minimal side effects (15). Thus, the improved SI of M_4N in comparison to that of NDGA may make M_4N a more promising antiviral candidate for further evaluation.

In summary, we have described that treatment with NDGA, and its derivative M_4N, reduced WNV and ZIKV multiplication. Although further studies of its potential cytotoxic effects in infected hosts are needed, our results together with the clinical safety record of M_4N point for the first time to this compound as an interesting candidate for antiviral design against medically relevant flaviviruses.

MATERIALS AND METHODS

Cells, viruses, infections, and virus titrations. All infectious virus manipulations were conducted in biosafety level 3 (BSL3) facilities. Vero (CCL-81) cells (ATCC) were grown at 37°C in a 5% CO_2 atmosphere in Eagle's minimal essential medium (EMEM; Lonza, Verviers, Belgium) supplemented with 2 mM glutamine, penicillin-streptomycin, and 5% fetal bovine serum. HeLa cells stably transfected with a plasmid encoding the last 25 amino acids of the WNV NY99 C protein followed by the sequence of premembrane/membrane (prM) and envelope (E) proteins (HeLa3-WNV cells) were grown in complete culture medium supplemented with 500 μg/ml G418 (18). The origin and passage history of the WNV lineage 1 strain NY99 has been previously described (18, 41). WNV lineage 2 isolate SRB-Novi Sad/12 corresponds to a virus isolated from a goshawk found dead in Serbia in 2012 (42). The American strain of ZIKV PA259459 corresponds to a virus isolated from an infected human in Panama in 2015 and was kindly provided by R. B. Tesh (World Reference Center for Emerging Viruses and Arboviruses, Galveston, TX). For infections in liquid medium, the viral inoculum was incubated with cell monolayers for 1 h at 37°C, and then the inoculum was removed and fresh medium containing 1% fetal bovine serum was added. The viral titer was determined by plaque assay in semisolid agarose medium (43). The multiplicity of infection (MOI) used in each experiment was expressed as PFU per cell as is indicated in the corresponding figure legend.

Drug treatments. NDGA, M_4N, zileuton, fatostatin, resveratrol, and BFA were from Sigma (St. Louis, MO). PF-429242 was from CliniSciences (Nanterre, France). Unless otherwise specified, drugs were added after the first hour of infection when viral inoculum was replaced by culture medium containing 1% fetal bovine serum. Control cells were treated in parallel with the same amount of drug vehicle (dimethyl sulfoxide [DMSO]). Drug toxicity was examined by measuring the cellular ATP content with the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI).

Antibodies. Mouse monoclonal antibody 3.67G and rabbit polyclonal antiserum PA1-4139 against the WNV E protein were from Millipore (Temecula, CA) and Thermo Fisher (Rockford, IL), respectively. Mouse monoclonal antibody against β-actin (Sigma) and mouse monoclonal antibody J2 against dsRNA (English & Scientific Consulting, Hungary) were also used. Anti-mouse IgG coupled to Alexa Fluor 488 (Life Technologies, Carlsbad, CA), anti-mouse IgG coupled to horseradish peroxidase (Sigma), and anti-rabbit IgG (Dako, Glostrup, Denmark) coupled to horseradish were used as secondary antibodies.

Confocal microscopy. Immunofluorescence and confocal microscopy were performed as described previously (17, 44). Nuclei were stained with To-Pro-3 (Life Technologies), and images were acquired using a Leica TCS SPE confocal laser scanning microscope and an HCX PL Apo 63×/1.4 oil immersion objective. Fluorescence quantification was performed using ImageJ software (https://imagej.nih.gov/ij/).

Enzyme-linked immunodot assay. The amount of E protein released to the culture medium by HeLa-3-WNV cells was determined using 3.67G monoclonal anti-E antibody and anti-mouse or anti-rabbit horseradish-coupled secondary antibodies (18, 19).

Western blot. The amount of E protein within infected cells was determined using the PA1-4139 rabbit antiserum against this protein following a previously published protocol (17).

Quantitative PCR. Viral RNA was extracted from the supernatant of infected cultures with the SpeedTools RNA virus extraction kit (Biotools B&M Labs, S.A., Madrid, Spain). For quantification of cell-associated viral RNA, supernatants from infected cells were removed and total RNA was extracted from cell monolayers using TRIzol reagent (Life Technologies). The amount of viral RNA was determined by real-time fluorogenic reverse transcriptase PCR (RT-PCR) according to previously published protocols (44, 45). As a control, the amount of rRNA 18S in RNA samples from cell monolayers was determined as described previously (46). Cell-associated viral RNA copies were normalized to this internal control. The number of viral RNA copies is given as the number of genomic equivalents corresponding to the number of PFU per milliliter by comparison with the amount of RNA extracted from previously titrated samples (47).

Data analysis. Data are presented as means ± standard deviations (SDs). Analysis of the variance (ANOVA) and Student's test were performed using SPSS 19 (SPSS Inc., Chicago, IL). Bonferroni's correction was applied for multiple comparisons. Statistically significant differences are indicated by one asterisk for a P value of <0.05 or two asterisks (**) for a P value of <0.005.

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DISCUSSION

The identification of viral and host processes that control the biology of flaviviruses such as WNV, USUV and ZIKV is important to improve, among other aspects, the design of specific innovative antiviral strategies

Cell lipid content alteration induced by WNV infection.

As explained in the introduction, flaviviruses depend on cellular factors to complete their life cycle, which is intimately associated to host cell lipids. Nowadays it is known that flaviviruses induce Intracellular membrane rearrangements to develop adequate platforms for viral replication and particle biogenesis. Moreover, flaviviruses dramatically orchestrate a profound reorganization of the host cell lipid metabolism to adequate cellular functions to their own requirements (Figure 9). Consistently, the role of specific lipid classes in flavivirus infections has been recently addressed. So that, among other features, it is already known that fatty acid synthesis is linked to viral replication, that phosphatidylserine and phosphatidylethanolamine are involved on viral entry, that sphingolipids play a key role on virus assembly and pathogenesis, and that cholesterol is essential for innate immunity evasion in flavivirus-infected cells (Martin-Acebes, Vazquez-Calvo, Saiz 2016).

In this line of research, to gain knowledge in the role of the cellular lipids in viral replication and to search for cellular antiviral targets, throughout the first investigations of this thesis (Article I), the specific modification that WNV exert over cell lipid metabolism was described for the first time. Thereby, and consistently with that previously reported for DENV (Perera et al., 2012), where about 15% of the lipids detected were changed between infected and non-infected insect cells, it was observed that in WNV-infected mammalian cells about 33% of the molecular species detected were significantly increased. In this way, an enhancement in the cellular content of glycerophospholipids (GPLs) and sphingolipids (SLs), remarkably of phosphatidylcholine (PC), lysophospholipids (lyso-PC), sphingomyelin (SM), and ceramide (CER) was observed (Fig. 1, Article I). At this point, it should be noted that CER provides the building block for the synthesis of more complex sphingolipids and, in turn, constitutes the central core of SM, and thus, both species can be interconverted by cellular enzymes (Figure 6) (Gault, Obeid et al. 2010; Merrill 2011). The observation that both CER and SM are increased in WNV-infected cells suggests that the first is the result of *de novo* synthesis rather than the degradation of SM. Although the lipolytic pathway of ceramide production via the catabolism of more complex SLs could also contribute to a rise in ceramide levels in flavivirus infected cells (Merrill 2011; Aktepe, Pham et al. 2015).

On the other hand, the fact that all enriched lipids, with the exception of PC (a cylindrical lipid), were conical strengthens their role in the establishment and maintenance of the cellular membrane curvature needed for the assembly of the viral replication complex (). Likewise, as described for DENV (Perera et al., 2012), the observed enrichment in unsaturated PC may be related with increasing the membrane's fluidity. A similar increase in SLs to the one observed here have also been documented for DENV and HCV (Diamond et al., 2010; Hirata et al., 2012; Perera et al., 2012). Therefore, overall, the lipid changes detected in WNV-infected cells resemble those derived from the infection with other member of the family *Flaviviridae* and point to common evolutionarily conserved ancient lipid requirements in this viral family (Martin-Acebes, Vazquez-Calvo, Saiz 2016).

The lipid composition of WNV envelope.

The lipid composition of cell cultured produced WNV virions and non-infectious RSPs were very similar (Fig. 4, Article 1), with a common reduction of PC and an enrichment of SM. This fact reinforces the utility of the RSPs for the study of the virus biogenesis without the need of BSL-3 facilities. Nevertheless, p-PC appeared to be enriched in virions compared to RSP. The differences found between natural virions and RSPs size (50nm and 30nm respectively), could be explained by these variations on the lipid membranes compositions. It has been demonstrated that vesicles formed with p-PC are larger than those formed with PC (Hermetter, Lohner et al. 1985).

The way different viruses make up their lipid membranes is quite diverse. For instances, HIV-1 and influenza virus show an increase in SL when compared with cellular membranes (Brugger et al., 2006; Gerl et al., 2012), rhabdoviruses and alphaviruses exert little lipid selection when acquiring their envelopes (Kalvodova et al., 2009), while others, such as HCV, have envelopes that resemble the composition of low and very low density lipoproteins (Merz et al., 2011). Our lipidomic analysis indicate that the envelopes of both WNV virions and RSPs are enriched in SM and exhibit a reduction in the content of PC compared to that of total cellular membranes. These features are characteristics of liquid ordered membrane microdomains or membrane rafts (Brugger e tal., 2006; Gerl et al., 2012; Trajkovic et al., 2008; Wubbolts et al., 2008), which are usually cholesterol enriched, including those located in the ER (Browman et al., 2008), and thus point to a role of these microdomains in flavivirus replication. In this sense, an enrichment of cholesterol is also observed in the envelope of bovine viral diarrhea virus (BVDV), a *Pestivirus* that also belongs to the *Flaviviriade* family (Callens, Brugger et al. 2016). Similarly to the WNV envelope, and consistent with our results, the pestivirus envelope is also

enriched in SLs (i.e. sphingomyelin) and showed a reduction of phosphatidylcholine. Thus, our results are consistent with common lipid selection requirement for the envelopment of *Pestivirus* and *Flavivirus*.

Flaviviral biogenesis: the role of the nSMase

As mentioned above, both CER and SM were increased in WNV-infected cells, which point to de *novo* synthesis of the later. Moreover, the fact that dhCER, an intermediate of SL biosynthetic pathway (Merrill AH, Jr 2011), was increased in WNV-infected cells also suggests *de novo* synthesis of SL, as observed in other viruses (Hirata et al., 2012; Perera et al., 2012). However, pharmacological inhibition and RNA interference of nSMase2, which hydrolyzes SM to render CER, diminished the release of RSPs into the medium (Fig. 5, Article I), and drastically reduced the production of infectious flavivirus, namely WNV and USUV (Fig. 6, Article I). These data point to nSMase2 as an important player in flavivirus biogenesis, in contrast to that observed for the alphavirus SINV, which did not require the conversion of SM to CER since lipids selection is not needed during its biogenesis (Kalvodova et al., 2009). All these data suggest that different SL-related metabolic pathways (*de novo* synthesis and hydrolysis) are functional for the production of CER in flavivirus infected cells. In addition, other factors such as induction of ER stress by activation of the unfolded protein response (Blázquez et al., 2014) could contribute to CER production in WNV-infected cells.

In brief, data obtained during this investigations (**Article I**) evidence the manipulation of the cellular lipid metabolism during WNV infection and the link between SL metabolism and flavivirus biogenesis, and mark out possible druggable targets for new antiviral strategies.

ACC inhibitors effect during flaviviral infection.

It is well known that the first steps of lipid biogenesis involve the synthesis and elongation of fatty acids, which are necessary for the synthesis of more-complex lipids, hence, making fatty acid metabolism a key target for antiviral therapy (Heaton et al., 2010; Munger et al., 2008, Nchoutmboube et al., 2013). In fact, the pharmacological blockage of the fatty acid synthase FASN (which catalyzes the synthesis of palmitate from acetyl-CoA, and malonyl-CoA into long-chain saturated fatty acids) reduce flaviviral replication (Martín-Acebes et al., 2011; Heaton et al., 2010). The enzyme preceding FASN in the fatty acid biosynthetic route is the ACC, which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA and, thus, ACC is currently being evaluated as a therapeutic target for different disorders including cancers, obesity, diabetes and even viral infections (Zu et al., 2013; Bourbeau MP and Bartberger MD 2015).

With this background and based on our previous results, the involvement of ACC in the replication of WNV, and other related flaviviruses, was evaluated (**Article II**). At this point, it was known that the ACC inhibitor TOFA reduced the synthesis of multiple cellular lipids, including some of those described by us in this thesis (**Article I**) to be involved in WNV infection such as GPLs, SLs and CHOL (Mackenzie, Khromykh et al. 2007; Martin-Acebes, Merino-Ramos et al. 2014). Therefore, the effect of TOFA on the infection of WNV was assayed. Lipidomic analysis of TOFA-treated cells showed that the drug reduces the cellular content of multiple lipids, including those directly implicated in the WNV life cycle (Fig. 1, Article II). Consequently, TOFA treatment produces a dose-dependent inhibition of WNV multiplication in different mammalian cells without exerting a toxic effect at the concentration assayed (Fig. 2, Article II), and inhibits genome replication (Fig. 3, Article II), more probably due to an impairment of the proper replication complex assembly caused by the lack of the necessary lipids. Similar reduction in viral multiplication was observed in USUV infected cells (Fig. 1, Article II). In addition, lipidomic analysis of WNV infected cells treated with TOFA showed a significant reduction in the content of CHOL, CER, hexCER, PC, PE and PS when compared with untreated cells (Fig. 4, Article II), thus indicating that the lipid changes induced by TOFA were similar in both WNV-infected and uninfected cells. Furthermore, treatment with another ACC inhibitor, MEDICA 16, also inhibited WNV and USUV infection (Fig. 5, Article II). However, the mechanism of action of both drugs seems to be different, as MEDICA 16 mainly affect the production of infectious virions and not the RNA synthesis. These may be explained because replication and assembly of viral particles are coupled processes in flaviviruses (Apte-Sengupta, Sirohi et al. 2014) and, thus, small differences on the alteration of the cellular lipid content specific for each inhibitor could result in the inhibition differences observed.

In brief, as demonstrated in our first investigations (**Article I**), data from this second work (**Article II**) confirm the role of lipogenesis during WNV infection and validate the ACC as a druggable cellular target suitable for broad-spectrum antiviral development against WNV and other flaviviruses.

SREBP pathway inhibitors effect during flaviviral infection.

In relation with these previous investigations (**Articles I and II**), it has been reported that the hypolipidemic drug NDGA inhibits the replication of viruses from different families (Chen, Teng et al. 1998; Craigo, Callahan et al. 2000; Hwu, Hsu et al. 2008; Khanna, Dalby et al. 2008; Pollara, Laster et al. 2010), including some from the *Flaviviridae* family, such as DENV (Soto-Acosta, Bautista-Carbajal et al. 2014) and HCV (Syed and Siddiqui 2011). NDGA, a phenolic

compound, is the main metabolite of the desert shrub *Larrea tridentate*, and is currently being evaluated to treat different illnesses, such as diabetes, pain, inflammation, infertility, rheumatism, arthritis, gallbladder and kidney stones (Arteaga, Andrade-Cetto et al. 2005; Lu, Nurko et al. 2010). Even more, a synthetic methylated derivative of NDGA, termed tetra-O-Methyl Nordihydroguaiaretic acid (M_4N), which is structurally highly similar to its precursor, is currently in Phase I/II clinical trials in patients with advanced cancer (Chen 2009; Grossman, Ye et al. 2012; Kimura and Huang 2016). The antiviral effect of NDGA has been related to its ability to disturb the lipid metabolism by interfering with the sterol regulatory element binding proteins (SREBP) pathway, since the SREBPS constitute the major family of transcription factors activating the expression of genes involved in biosynthesis of lipids (Goldstein, DeBose-Boyd et al. 2006; Tang, Li et al. 2011).

With this base, and following the research line of this thesis, the potential antiviral effect of drugs interfering with SREBP pathway against two flavivirus, WNV and ZIKV, was evaluated (**Article III**). NDGA and its synthetic methylated derivative M_4N inhibited the multiplication of WNV lineage 1 and 2 strains (Fig 1, Article III) and ZIKV (Fig 4, Article III) in mammalian cells without exerting noticeable cell toxicity. Moreover, the determined CC_{50} values of each compound evidenced a reduced toxicity of M_4N. These results are in agreement with previous data reporting that the median lethal dose of M_4N for mice is much higher than that of NDGA (> 1000 mg/kg *vs* 75 mg/kg) (Kimura and Huang 2016), and that M_4N presented low toxicity for human usage with minimal side-effects (Grossman, Ye et al. 2012). On the other hand, quantitative RT-PCR and immunofluorescence analyses demonstrated that both drugs, NDGA and M_4N, impaired the replication of WNV genome, but did not affect virion morphogenesis and egress, as no significant reduction of virus-like particles production was noticed (Fig 2, Article III). Even more, in support of the hypothesis pointing to an inhibition of the SREBP pathway as the mechanism behind the antiviral activity of NDGA (Syed and Siddiqui 2011; Soto-Acosta, Bautista-Carbajal et al. 2014), the here obtained experimental data show that a panel of structurally unrelated inhibitors of this pathway (Resveratrol, Fatostatin, and PF-429242) also reduced the multiplication of both WNV and ZIKV (Fig 3, Article III).

In brief, in agreement with our previous observations about the role of lipids in flavivirus replication (**Article I and II**), the results of this third investigation (**Article III**) point to drugs targeting the cellular SREBP pathway as new additional options to fight flaviviral infections.

Feasibility of lipid-targeted antiviral strategies

A great effort aimed to decipher the molecular biology of flaviviruses is being made by the scientific community. Therefore, within the last years, important attention has been paid to WNV and its interaction with the host immune system (Brinton 2013; Suthar, Diamond et al. 2013), however, as commented in the Introduction, no licensed vaccine or therapy for human use against neither WNV, nor USUV, nor ZIKV is available yet. Overall, during this thesis, it has been demonstrated that flavivirus multiplication is closely related to lipid metabolism, as well as the important role that some lipids play in the replication and biogenesis of different flaviviruses. This analysis has allowed determining the key role of certain cellular enzymes and pathways and, thus, evidencing the potential of drugs targeting them as antivirals to fight flavivirus infections.

The possibility of using lipid synthesis inhibitors as antiviral compounds should be disclose, as is still a controversial subject. On the one hand, it has to be consider that metabolic pathway manipulation could induce multiple side effects *in vivo.* On the other hand, antivirals currently available mainly target other major biosynthetic pathways, like nucleotide biosynthesis. In addition, it is a fact that commonly used drugs interfere with the lipid metabolism or are lipid related molecules, such as the cyclooxygenase-2 inhibitors (aspirin) or the anti-inflammatories (ibuprofen). Remarkably, some of the host targets that we have focused on during this research are already being investigated or used for the treatment of other pathologies. This is the case of the ACC, which is being investigated by the pharmaceutical industry for the development of drugs for diabetes, obesity and cancers due to its rate-limiting role in fatty acid synthesis (Zu, Zhong et al. 2013; Bourbeau and Bartberger 2015). In mammals, ACC expression and activity is regulated at transcriptional, posttranslational, and metabolite-allosteric levels. At the transcriptional level, ACC1/2 expression is regulated by the SREBP-1. Because of this tight relation and because aberrant SREBP activity has been linked to metabolic disease states, such as obesity, fatty liver, insulin resistance, hyperlipidemia, and atherosclerosis (Tang, Li et al. 2011; Uesugi 2013). Thus, ACC and SREBP constitute major metabolic regulators under clinical evaluation. Furthermore, SREBP is under investigation as well (Uesugi 2013; Xiao and Song 2013), it has been described that SREBP-2 is mainly responsible for the regulation of cholesterol related genes, such as HMG-CoA reductase, a rate-limiting enzyme in cholesterol synthesis, and low-density lipoprotein receptor (LDLR) gene (Moore, Rayner et al. 2010). Moreover, statins, the HMG-CoA reductase inhibitors that are widely used in human to lower cholesterol levels (Carroll, Lacher et al. 2005), have also been tested for HCV-antiviral properties (Ikeda, Abe et al. 2006; Delang, Paeshuyse et al. 2009;

Blanchet, Seidah et al. 2012). Similarly, the HMG-CoA reductase inhibitor lovastatin increased survival rates for all treatment regimens *in vivo* against DENV-2 infection (Martinez-Gutierrez, Correa-Londono et al. 2014), nevertheless, reduced viraemia could only be observed in pretreated animals. Considering the broad usage and good tolerability of statins, they could be candidates for an emergency prophylactic antiviral regimen. In any case, compared with DENV or HCV, the effort for WNV drug discovery has been much lower, more probably due to the relatively lower mobility and mortality rates of this virus, as only about 1% of cases develop severe neuroinvasive disease (encephalitis, meningitis or flaccid paralysis). However, it is quite probable that, due to the high homology among different flaviviruses, antivirals developed for one may potentially be useful for others. In this sense, ideally, the best antivirals would be repurposed drugs, as they have established safety and pharmacokinetic profiles; however, their activity spectrum should also be taken into account, being preferable drug candidates that can be active against the largest number of viruses. In relation with this, as the lipid requirement is quite conserved among viruses, lipid-based host target antiviral strategies could confer broad-spectrum antiviral activity. Furthermore, there is an obvious advantage of the lipid-based antiviral strategies over DAA; they are less likely to develop resistance due to viral mutations. However, we have to keep in mind that our main goal is to fight flaviviral infections, and that DAAs and host target antiviral strategies are not restrictive each other, and, thus, new antiviral strategies should be developed in combination and not just in monotherapy, as they would be much more effective against viral infections. In brief, it is reasonable to think that the drugs tested along this research could inhibit different circulating viruses, as well as (re)emerging viruses.

CONCLUSIONS

Las conclusiones obtenidas en esta tesis fueron las siguientes:

1. El contenido de una amplia variedad de metabolitos lipídicos se encuentra alterado en células infectadas con VNO.
2. Algunos glicerofosfolípidos (fosfatidilcolina, plasmalógenos y lisofosfolípidos) y esfingolípidos (ceramida, dihidroceramida y esfingomielina) se encuentran aumentados en células infectadas con VNO.
3. Las envueltas lipídicas de los viriones de VNO naturales y de las partículas virales recombinantes están enriquecidas en esfingolípidos (esfingomielina) y muestran niveles reducidos de fosfatidilcolina.
4. La inhibición de la esfingomielinasa neutra (la cual cataliza la hidrólisis de la esfingomielina a ceramida) reduce la liberación de viriones de flavivirus así como de partículas subvirales recombinantes.
5. El metabolismo de los glicerofosfolípidos y esfingolípidos está ligado a la infección por el VNO.
6. TOFA y MEDICA 26, ambos inhibidores de la acetil-CoA carboxilasa (ACC) inhiben la multiplicación del VNO y del virus Usutu mediante la reducción de la replicación del genoma viral.
7. El ácido nordihidroguaiaretico (NDGA) y su derivado metilado el tetra-*O*-metil nordihidroguaiaretico (M_4N) inhiben la infección del VNO y del virus de Zika.
8. Inhibidores de la vía de la proteína de unión a elementos reguladores del esterol (SREBP) que no están estructuralmente relacionados con el NDGA, como son el PF-429242 y la Fatostatina, reducen la multiplicación del VNO y del virus Zika.

The conclusions obtained with this thesis were the following:

1. The content of a wide variety of lipid metabolites is altered in WNV-infected cells.
2. Several glycerophospholipids (phosphatidylcholine, plasmalogens and lysophospholipids) and sphingolipids (ceramide, dihidroceramide and sphingomyelin) are increased in WNV-infected cells.
3. The lipid envelopes of natural WNV virions and recombinant virus-like particles are enriched in sphingolipids (sphingomyelin) and show reduced levels of phosphatidylcholine.
4. Inhibition of neutral sphingomyelinase (which catalyzes the hydrolysis of sphingomyelin into ceramide) reduces the release of flavivirus virions as well as that of virus-like particles.
5. Glycerophospholipid and sphingolipid metabolism is linked to WNV infection.
6. The acetyl-CoA carboxylase (ACC) inhibitors TOFA and MEDICA 16 inhibit the multiplication of WNV and USUV by reducing viral genome replication.
7. The nordihydroguaiaretic acid (NDGA) and its methylated derivative tetra-*O*-methyl nordihydroguaiaretic (M_4N), inhibit the infection of WNV and ZIKV.
8. Inhibitors of the sterol regulatory element binding proteins (SREBPs) pathway that are structurally unrelated to NDGA, such as PF-429242 and Fatostatin, reduce WNV and ZIKV multiplication.

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APPENDIX I

LIST OF THE PhD FELLOW PUBLICATIONS

1. **Merino-Ramos T**, de Oya NJ, Saiz JC, Martín-Acebes MA (2017). "Antiviral activity of nordihydroguaiaretic acid and its derivative tetra-O-methyl 2 nordihydroguaiaretic acid against West Nile virus and Zika virus." Antimicrob Agents and Chemother. 2017 May 15. pii: AAC.00376-17. doi: 10.1128/AAC.00376-17

2. Saiz JC, Blázquez AB, De Oya NJ, **Merino-Ramos T**, Martín-Acebes MA, Escribano-Romero E, Vázquez-Calvo Á. "Response: Commentary: Zika Virus: the Latest Newcomer."Front Microbiol. 2016 Sep 7;7:1398. doi: 10.3389/fmicb.2016.01398.

3. Saiz JC, Vázquez-Calvo Á, Blázquez AB, **Merino-Ramos T**, Escribano-Romero E, Martín-Acebes MA (2016). "Zika Virus: the Latest Newcomer." Front Microbiol. 2016 Apr 19;7:496. doi: 10.3389/fmicb.2016.00496. eCollection 2016. Review.

4. Martín-Acebes MA, Blázquez AB, Cañas-Arranz R, Vázquez-Calvo Á, **Merino-Ramos T**, Escribano-Romero E, Sobrino F, Saiz JC (2016). "A recombinant DNA vaccine protects mice deficient in the alpha/beta interferon receptor against lethal challenge with Usutu virus." Vaccine. 2016 Apr 19;34(18):2066-73. doi: 10.1016/j.vaccine.2016.03.015. Epub 2016 Mar 15.

5. **Merino-Ramos T**, Martín-Acebes MA, Casal J, Saiz JC, Loza-Rubio E (2016). "Prevalence of Hepatitis E Virus (HEV) Antibodies in Mexican Pigs." Food Environ Virol. 2016 Feb 15.

6. **Merino-Ramos T**, Vázquez-Calvo Á, Casas J, Sobrino F, Saiz JC, Martín-Acebes MA (2015). "Modification of the Host Cell Lipid Metabolism Induced by Hypolipidemic Drugs Targeting the Acetyl Coenzyme A Carboxylase Impairs West Nile Virus Replication." Antimicrob Agents Chemother. 2015 Oct 26;60(1):307-15. doi: 10.1128/AAC.01578-15.

7. Escribano-Romero E, Lupulović D, **Merino-Ramos T**, Blázquez AB, Lazić G, Lazić S, Saiz JC, Petrović T (2015). "West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia." Vet Microbiol. 2015 Apr 17;176(3-4):365-9. doi: 10.1016/j.vetmic.2015.02.005. Epub 2015 Feb 14.

8. **Merino-Ramos T**, Blázquez AB, Escribano-Romero E, Cañas-Arranz R, Sobrino F, Saiz JC, Martín-Acebes MA (2014). "Protection of a single dose west nile virus recombinant subviral particle vaccine against lineage 1 or 2 strains and analysis of the cross-reactivity with Usutu virus." PLoS One. 2014 Sep 17;9(9):e108056. doi: 10.1371/journal.pone.0108056. eCollection 2014.

9. Martín-Acebes MA, **Merino-Ramos T**, Blázquez AB, Casas J, Escribano-Romero E, Sobrino F, Saiz JC (2014). "The composition of West Nile virus lipid envelope unveils a role of sphingolipid metabolism in flavivirus biogenesis." J Virol. 2014 Oct;88(20):12041-54. doi: 10.1128/JVI.02061-14. Epub 2014 Aug 13.

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